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The role of CD28 in T cell activation

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THE ROLE OF CD28 IN T CELL ACTIVATION.

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THIS DISSERTATION IS DEDICATED TO
JIM AND MHAIRI WILSON
FOR THEIR LOVE AND SUPPORT.

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Contents

Summary

This research has investigated the role of CD28 in T cell activation. These studies have clearly demonstrated that CD28 plays a major role in providing signals to T cells which in conjunction with the T cell receptor (TCR) result in interleukin -2 (IL-2) production and T cell proliferation. Kinetic analysis revealed that costimulatory signals through CD28 are required within 6 hours of TCR engagement and T cells were unresponsive after this time. The surface expression of CD28 was found to be downregulated following ligation by CD80 but was re-expresssed following T cell activation. CD28 ligation by CD80 on previously activated T cells resulted in augmented T cell proliferation suggesting that CD28 signalling may prolong and/or enhance T cell responses, however this enhancement did not appear to involve IL-2 production.

Studies of CD28 signalling was found to involve the enzyme phosphatidylinositol 3-kinase (PI3-kinase) and inhibition of this enzyme by wortmannin prevented IL-2 production and T cell proliferation. This inhibitory effect was not due to inhibiting IL-2R signalling. In contrast, the augmented proliferation by previously activated T cells, in response to CD28 ligation was found be insensitive to wortmannin suggesting PI3-kinase was not required.

Comparison of the functional effects of the two CD28 ligands, CD80 and CD86 showed that both ligands could costimulate T cell proliferation in conjunction with an anti-CD3 mAb and similarly both ligands augmented proliferation of activated T cells. These studies highlight the role of CD28 in T cell function and suggest that signals through CD28 may differ depending on the state of T cell activation.

Abbreviations

AIDS	Acquired immune deficiency syndrome.
AP-1	Activator protein 1.
APC	Antigen presenting cell.
ATCC	American Type Culture Collection.
Ca^{++}	Calcium ions.
CD	Cluster of differentiation.
CD28RE/RC	CD28 response element/response complex.
cDNA	Complementary deoxyribonucleic acid.
CDR	Complementarity determining region.
CHO cells	Chinese Hamster Ovary cells.
CsA	Cyclosporin A.
CTL	Cytotoxic T lymphocyte.
CTLL-2	cytotoxic T cell line 2
CTLA-4	Cytolytic T lymphocyte associated antigen -4.
DAG	1, 2, diacylglycerol.
DEPC	Diethylpyrocarbonate.
DMEM	Dulbecco's minimal essential medium.
EAE	Eperimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid.
ELISA	Enzyme-linked immunosorbent assay.
ER	Endoplasmic reticulum.
FACS	Fluorescence activated cell sorter.
FCS	Foetal calf serum.
FITC	Fluorescein isothiocyanate.
FL1	Fluorescence intensity
FSC	Forward scatter.
GAPDH	Glyceraldehyde -3-phosphate dehydrogenase.
HIV	Human immunodeficiency virus.
ICAM	Intercellular adhesion molecule.
IFN	Interferon.
Ig	Immunoglobulin.
IL	Interleukin.
IL-2R	Interleukin - 2 receptor.
IP_3	Inositol 1,4,5-trisphosphate.
LCMV	Lymphochoriomeningitis virus.
LFA	Lymphocyte function-associated antigen.

LMP	Large multifunctional proteinase.
mAb	Monoclonal antibody.
MFI	Mean fluorescence intensity.
MHC	Major Histocompatibility complex.
MLR	Mixed lymphocyte reaction.
Mls	Minor lymphocyte stimulating.
mRNA	Messenger ribonucleic acid.
NFAT	Nuclear Factor of activated T cells.
NF-kB	Nuclear factor-kappa B.
NOD	Non-obese diabetes.
NIBSC	National Institute for Biological Standards and Controls
PBMC	Peripheral blood mononuclear cells.
PBS	Phosphate buffered saline.
PHA	Phytohaemagglutinin.
PI3-kinase	Phosphatidylinositol 3 -kinase.
PIP_2	Phosphatidylinositol 4,5 bisphosphate.
PKC	Protein kinase C.
PLC	Phospholipase C.
PMA	Phorbol myristate acetate.
PTK	Protein tyrosine kinase.
PTP	Phosphotyrosine phosphatase.
rhIL-2	Recombinant human interleukin -2.
RT-PCR	Reverse transcription-polymerase chain reaction.
SAS	Saturated ammonium sulphate.
SEB	Staphylococcal enterotoxin B.
SH	Src homology.
SLE	Systemic lupus erythematosus.
SSC	Side scatter.
TAM	Tyrosine based activation motif.
TAP	Transporter associated with antigen processing.
TCR	T cell receptor.
Th	T helper.

CHAPTER 1.

INTRODUCTION.

The ability of an organism to defend itself from infectious attack is essential for its survival. Defence mechanisms can operate at a number of different levels with the key component being the immune system. The first line of defence is the skin and other, mainly internal, surfaces which act as a barrier to invasion by potentially toxic substances/organisms. The next level of protection is the innate immune system made up of phagocytic cells which can engulf and eliminate any foreign substances (harmful or not) they come into contact with. The most sophisticated defence mechanism is the acquired immune system which is capable of detecting and destroying foreign invaders of the host but which has a memory component to ensure future attacks are repelled more quickly. The complexity of the immune system ensures that an appropriate response both in intensity and duration is mounted against potential pathogens.

Unfortunately the immune system is not foolproof and there are many examples of diseases which involve its malfunction. The most obvious are the autoimmune diseases such as rheumatoid arthritis and multiple sclerosis where one of the clinical symptoms is the attack of healthy host cells by their own immune system. Other diseases, such as cancer, show the immune system failing to detect and subsequently destroy infected/abnormal cells. Sometimes even the immune system is unable to protect itself, as in Acquired Immune Deficiency Syndrome (AIDS) where CD4 T cells are the target cells of the human immunodeficiency virus (HIV) virus. An efficiently functioning immune system can also, in exceptional circumstances, be deleterious. For example, tissue/organ transplantation is often used to prolong the survival of an individual. However, an efficient immune system will reject the foreign tissue counteracting the benefits gained by the surgical intervention.

All the above diseases/disorders have a common factor - an inappropriately functioning acquired immune system. By determining how the acquired immune system functions under normal circumstances it may be possible to use the knowledge gained to manipulate the immune system. Immunotherapy could then be used as a potential treatment of such diseases. However determining how the highly complex acquired immune system functions normally is an immense task.

The acquired immune system can mount an appropriate response both in terms of duration and intensity depending on the seriousness of the attack on the organism. It is this ability to determine the correct response to individual

circumstances which makes this a highly complex defence system requiring tight control mechanisms to ensure proper functioning. However only a few different types of cells are involved - T lymphocytes (T cells), B lymphocytes (B cells) and a number of different antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells. All these cells have different functions and interactions between these cells are required to ensure a successful response by the immune system.

The ability to recognise foreign antigen is a function of both B cells and T cells. B cells are able to recognise foreign antigen directly via the specific antibody expressed on the B cell surface. In contrast T cells require interaction with antigen presenting cells which display foreign antigen on their surface for recognition to occur. There are also interactions between T cells and B cells with B cells requiring "T cell help" to mount a full B cell immune response. Since the aims of this research were to study the role of CD28 in T cell activation, the role of B cells in the immune system will not be considered further.

T lymphocytes and their receptors.

T cells play an important role in the acquired immune response by being able to recognise foreign antigen and initiate a cascade of events which results in the elimination of the antigen (Ashwell, 1990). It is essential that T cells perform their recognition function correctly. Failure to detect a foreign pathogen or recognition of a self antigen could have deleterious consequences. How T cells function under normal circumstances is still the subject of research but considerable information is available about T cell recognition of foreign antigen.

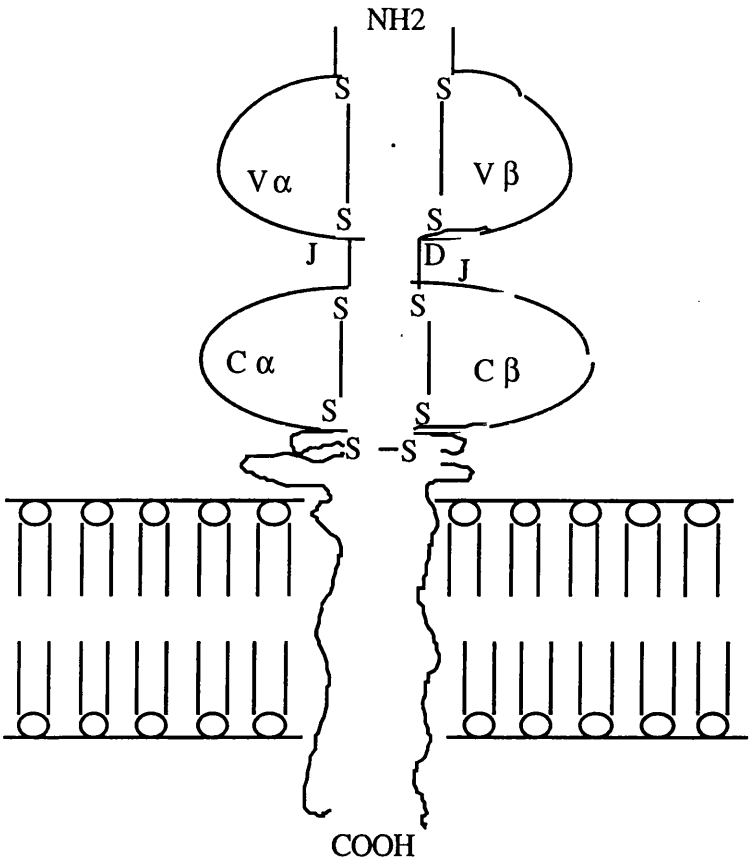
The T cell receptor (TCR).
(reviewed by Ashwell, 1990; Lefranc, 1990)

The site of foreign antigenic recognition on T cells is the T cell receptor (TCR) which is a heterodimeric structure of disulphide linked α and β chains (or δ and γ chains in approximately 3% of T cells) with a single antigen recognition site (Figure 1.1a). The overall structure of the TCR on each T cell is similar but the TCR on an individual T cell has its own unique antigen

binding specificity. In order to generate these two requirements (similar overall structure but unique binding site) the α and β chains of the TCR are encoded by a number of genes. The constant region genes are responsible for the overall similarity in structure of the TCR. The variable and junction genes are responsible for generating the unique binding site of the TCR. The number of variable and junction genes for each of the TCR chains is large (60 variable genes and 15 junction genes for the β chain and 80 variable and 80 junction genes for the α chain) (Wilson et al., 1988, Lai et al., 1988). The various combination of these genes for each chain (approximately 1500 for the β chain and 6400 for the α chain) and the combinations of α and β chains which can associate will generate a large range of TCRs with different antigen binding sites. However the overall diversity of the TCR required to ensure that all potential antigens can be recognised could not be generated solely by the different combinations of these genes. Additional diversity is generated by variable splicing of the junction and variable genes.

The TCR does not exist in isolation on the T cell surface. It is associated non-covalently with a number of invariant chains (γ, δ, ε, ζ and η) which make up the CD3 complex (Figure 1.1b) (Clevers et al., 1988). Although all these chains are associated with the TCR they play no role in determining antigen specificity - this is determined by the αβ chains. However the CD3 complex does have an important role to play in T cell function - it is essential for intracellular signalling by the TCR which will be discussed later.

a)



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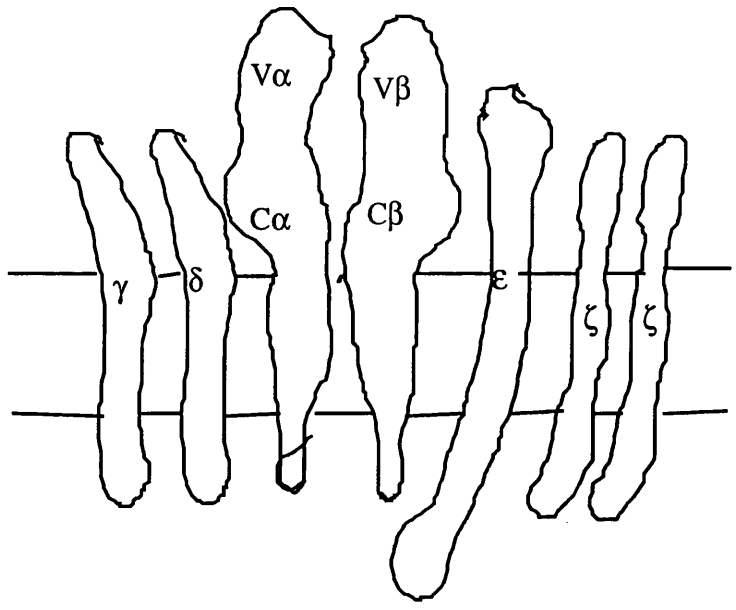


Figure 1.1. T cell receptor structure.

a) The TCR consists of a heterodimeric structure of disulphide linked α and β chains composed of constant, junction and variable domains.

b) The TCR/CD3 complex comprises the TCR non-covalently linked to at least four different invariant chains.

(From Lefranc 1990).

Unlike B cells, T cells are unable to interact directly with antigen - the foreign antigen must be "presented" to them by antigen presenting cells (Grey and Chesnut, 1985). It is essential that T cells only interact with foreign antigen and do not generate an immune response to self antigens. An understanding of how APCs process and present antigen on their surface has provided insights into how T cells are able to distinguish between foreign and self antigens.

Antigen processing and presentation

The interaction between T cells and APCs requires both cells to come into close contact to allow the TCR of the T cell to recognise the foreign antigen expressed on the surface of the APC. As the foreign antigen may be large, this close contact is achieved by the APCs engulfing the foreign antigen, processing it internally, and displaying antigenic peptide on their surface. In order for the T cell to be able to recognise "foreign" antigens it has to be able to recognise " self". This is achieved by the TCR of the T cell interacting with an antigen-Major Histocompatibility complex (MHC) complex on the surface of the APC.

MHC molecules are important surface markers which identify all host cells as "self". There are two types of MHC molecules, involved in antigen presentation, known as Class I and Class II (Hood et al., 1983). The distribution of these molecules is distinct with Class I found on all nucleated cells whereas MHC Class II is restricted to APCs and induced on activated T cells. Both Class I and Class II MHC molecules are each composed of two polypeptide chains (α and β) which show some sequence homology but have major differences in their structure. In Class I MHC molecules the α chain is much larger (molecular weight of 44kDa) than the β chain (known as β_2 - microglobulin with a molecular weight of 12kDa) whereas the α and β chains in Class II MHC molecules are similar in size (molecular weights of 34 and 29 kDa respectively). However both MHC molecules have a similar 3-dimensional structure composed of two alpha helices above a beta pleated sheet with a cleft being formed between the alpha helices (Bjorkman et al., 1987a; Bjorkman et al., 1987b; Brown et al., 1988). It is within this cleft that antigenic peptide binds and is presented for recognition by T cells. The size of this cleft differs between Class I and Class II MHC molecules with the cleft in Class I MHCs being "closed" at both ends whereas in Class II MHCs one end is "open". This difference affects the ability of the two types of MHC to bind

antigenic peptide - with MHC Class I binding peptides of 8 -10 amino acids whereas MHC Class II can bind larger peptides (15 - 24 amino acids) (Bjorkman et al., 1987a; Bjorkman et al., 1987b; Brown et al., 1988).

The antigenic peptides presented in Class I and Class II MHCs differ both in the source of the antigen and in the processing of the antigen to form peptides. Antigens presented in the context of MHC Class I molecules are derived from endogenous sources and are degraded by the large multifunctional proteinase (LMP) complex. The resulting peptides are delivered to the transporter associated with antigen processing (TAP) complex in the endoplasmic reticulum (ER) which transports them into the ER lumen where they bind to MHC Class I molecules and are exported to the cell surface (reviewed by Monaco, 1992). The peptides presented on MHC Class II are derived from extracellular sources and are endocytosed and degraded within endosomes and lysosomes. The MHC Class II molecules are synthesised within the ER but do not bind endogenous antigenic peptide (which is presented in the context of MHC Class I molecules) due to the presence of the invariant chain which loosely associates with the MHC $\alpha\beta$ heterodimer. Binding of the peptide occurs somewhere on the endocytic processing pathway and may differ depending on the cell type (reviewed by Neefjes and Ploegh, 1992).

As the TCR on the T cell interacts with both the MHC molecule and the antigen presented within the MHC, T cells are able to distinguish between cells which express "self" MHC containing self peptide and those expressing either foreign antigenic peptide within the self MHC or totally foreign cells which express "non-self" MHCs (as in organ transplantation) and respond appropriately. This ability of T cells to respond only to the presence of foreign antigen (either foreign antigenic peptide within self MHC or foreign MHC molecules) is a result of selection of T cells during development. The selection process results in the development of T cells which can recognise self MHC but are not self-reactive. The exact mechanism of this selection process is still being determined but considerable insight into the selection process during T cell development has been achieved.

T cell development.
(reviewed by Nikolic-Zugic, 1991).

T cells play a crucial role in the recognition of foreign antigen and the initiation of a cascade of events which eventually result in the destruction of the foreign pathogen. It is essential that the T cells which develop in the thymus and are transported to the periphery to carry out their immunosurveillance task are capable of detecting foreign antigens and are not self reactive. Failure of the selection process during T cell development could have disastrous consequences - pathogens which were not recognised could cause major damage and recognition of self antigens could result in an inappropriate immune response to host cells.

T cell development is a complex process in which more than 90% of the thymocytes are eliminated and do not mature into T cells. The T cell precursors are transported from the bone marrow to the thymus where a developmental process occurs. The most critical event in thymocyte maturation is the expression of the TCR which will recognise a specific antigen in the context of either MHC Class I or Class II. The co-receptors CD4 and CD8 are also both expressed on the thymocytes which are subject to intrathymic selection.

As the TCR is generated by random rearrangements of the numerous constant and variable genes of the α and β chains as well as variable splicing of these genes, the number of potential TCRs produced is enormous. Some of the TCRs generated may not be functional and some may be self - reactive. It is essential that thymocytes bearing these TCRs are eliminated. Although the exact mechanism of T cell selection is unknown it is thought to occur in two distinct stages - positive and negative selection - which occur in different regions of the thymus.

Positive selection is thought to occur via the cortical epithelial cells within the thymus which express an MHC - peptide complex. As the TCR expressed on each thymocyte will only recognise a specific MHC - peptide complex, it seems likely that only those thymocytes which recognise and bind to a particular MHC - peptide complex expressed by the cortical cells will be positively selected (Lo & Sprent 1986, Benoist & Mathis 1989). Those thymocytes which are unable to recognise and bind to a specific MHC-

peptide complex or do not express a functional TCR will be rejected and undergo programmed cell death (apoptosis).

Negative selection is thought to occur in the medulla of the thymus where the thymocytes interact with haemopoetic and possibly medullary epithelial cells (Lo & Spent 1986, Benoist & Mathis 1989). The T cells again interact with MHC - peptide complexes which may not necessarily be the same as during positive selection. Due to the specificity of the TCR for a particular MHC - peptide complex an inappropriate interaction with an another MHC - peptide complex or a high affinity interaction with the correct MHC- peptide complex is thought to lead to clonal deletion (Smith et al., 1989). This selection process which results in the elimination of self-reacting T cells is known as central tolerance.

The expression of CD4 and CD8 on the developing T cells is also considered to be involved in T cell selection. The differentiation of the developing thymocytes into either CD4 or CD8 T cells occurs in the thymus. There are two models of selection - the instructive model and the selective model. The instructive model suggests that the MHC restriction of the TCR expressed on the developing thymocyte determines the function of the T cell. This is thought to occur early in positive selection when the thymocyte expresses both CD4 and CD8. The extraneous CD4 or CD8 receptor is switched off following interaction of the thymocyte with a thymic stromal cell bearing the appropriate MHC restriction. The selective model suggests that positive selection occurs later when the thymocytes express either CD4 or CD8. Thymocytes only continue to develop if they interact with their appropriate thymic stromal cells with both the TCR and the correct co-receptor. There is evidence to support both models of selection and research suggesting that both processes may be involved (Chan et al., 1993).

Although the exact mechanism of T cell selection is still to be elucidated, the end result of T cell development in the thymus is the production of mature T cells. These T cells are then transported to the periphery where they carry out their immunological surveillance functions. A critical component of the immunosurveillance function of peripheral T cells is recognition of foreign antigens. This involves recognition of the foreign antigen presented by the MHC on the surface of the APC by the TCR of the T cell. The interaction between the TCR on the T cell and the MHC-antigen complex on the APC will now be examined.

TCR - MHC-antigen interactions

The interaction between the MHC/peptide complex and the TCR usually results in the antigenic fragment within the MHC cleft interacting with both the TCR α and β chains. As the number of T cells with the specific TCR recognition site for each antigen will be small, this will lead to a low frequency response - approximately 1 in 10,000 T cells will respond (Davis and Bjorkman, 1988). However there are occasions when a large T cell response can be generated. This occurs when "superantigens" (e.g. staphylococcal enterotoxins) are presented by the MHC to the T cells. The ability of "superantigens" to generate a large immune response is due to the way they bind to the MHC molecules and their interaction with the TCR. The superantigen is not processed by the APC to produce antigenic peptide - the whole protein binds to the MHC, probably to the outer surface and not in the normal binding cleft. This results in the interaction between the MHC/antigen complex and the TCR/CD3 complex occurring primarily with the Vβ region of the TCR (Dellabonna et al., 1990). The superantigen can therefore interact with all the TCRs bearing the appropriate Vβ region regardless of their other components. As there are a limited number of Vβ elements, this interaction results in a large proportion (5% - 40%) of the total T cell population being stimulated (Janeway et al., 1989).

CD4 T cells play an essential role in the initial recognition of foreign antigenic peptide. Interaction between the CD4 T cells and APCs results in the transcription of over 100 genes in the 2-3 weeks duration of the immune response (Ullman et al., 1990). The stimulation of CD4 T helper (T_h) cells results in their differentiation into two distinct sub populations - Type 1 T_h (T_h1) and Type 2 T_h (T_h2) cells each of which produces its own set of cytokines and mediate separate effector functions. (Mosmann and Coffman, 1989; Seder et al., 1994). T_h1 cells produce IL-2 and also γ-interferon which leads to the activation of granulocytes and macrophages and the induction of their phagocytic activity. T_h2 cells produce cytokines such as interleukin - 4 (IL-4), interleukin - 5 (IL-5) and interleukin - 10 (IL-10) which stimulate production of mast cells and eosinophils and in conjunction with antigen activate B cells to proliferate, differentiate and produce immunoglobulin. This activation of the immune defence system results ultimately in the destruction of the foreign antigen and the attainment of immunological memory so that if the same antigen is encountered again the immune response will be faster and of greater potency.

Adhesion molecules

Activation of T cells can only occur if sufficient TCRs on the cell surface become occupied with antigen. Antigen independent assistance given by a number of surface molecules on T cells is essential to establish contact between the T cells and the APCs (Figure 1.2). This adhesive function is necessary to bring the plasma membranes of the two cells in close contact. It also allows the T cell time to bring the TCR (by membrane diffusion) into contact with the antigen/MHC complex which may be present at low concentrations and stabilises this low affinity interaction (Makgoba et al., 1989). It has been demonstrated using human cytolytic T lymphocytes (CTL) that antigen -independent adhesion precedes antigen-specific recognition and T cell activation. By using blocking mAbs, it was possible to show that such adhesion is regulated by the cell surface molecules CD2, LFA-3 (CD58) and LFA-1 (CD11a/18) (Shaw et al., 1986).

CD2/CD58

CD2 is found on 95% of all T cells and its ligand CD58 is widely expressed. There is the possibility of a second ligand for CD2, as two mAbs are required to stimulate CD2 (Olive et al., 1986). It has been shown that CD59 which is widely expressed on both haemopoetic and non-haemopoetic human cells can bind to CD2 and may act as a second ligand for CD2 in humans (Hahn et al., 1992) whereas in mice CD48 has been identified as the possible second ligand (Kato et al., 1992). CD2 has been shown to stimulate T cells in conjunction with a primary signal via the TCR using monoclonal antibodies (mAbs) to CD2 or LFA3- Ig chimeric molecules to stimulate the CD2 on T cells (Carrera et al., 1988, Damle et al., 1992). However closer examination of the T cells stimulated by CD2 (in conjunction with TCR stimulation) showed that the T cells were predominantly memory cells and not naive T cells. CD2 may play a role in restimulation of T cells and not the primary activation of naive T cells. This may be due to the way CD2 signals. CD2 itself has no intrinsic signalling mechanism and has been shown to signal via the ζ chain of the TCR/CD3 complex (Howard et al., 1992). Activation of CD2 using either crosslinked CD58/Ig fusion proteins or anti-CD2 mAbs results in phospholipase Cγ1 (PLC) tyrosine phosphorylation and increased intracellular calcium concentrations (Kanner et al., 1992). This augmentation of the TCR signalling mechanism may be able to activate memory T cells but may be insufficient to fully activate naive T cells .

LFA-1/ICAM1

LFA-1 (CD11a/CD18) has also been shown to be involved in adhesion between T cells and APCs. There are three known ligands for LFA-1 i.e. ICAM-1(CD54), ICAM-2 (CD102) and ICAM-3 (CD 50) (Marlin and Springer, 1987; Fawcett et al., 1992). Interactions between LFA-1 and ICAM-1 have been shown to provide costimulatory signals which synergise with that provided via the TCR/CD3 complex (van Noesel et al., 1988; Van Seventer et al., 1992). However, as ICAM-1 is expressed at low levels on resting white blood cells, this interaction may not play a major part in T cell activation. ICAM-3 on the other hand is constitutively expressed on all leukocytes with the highest concentrations of mRNA found in antigen presenting cells such as monocytes/macrophages and B cells. As ICAM-3 is closely related to ICAM-1, with key residues in the LFA-1 binding site being conserved, it may be that ICAM-3 /LFA-1 interactions have an important role to play in T cell activation (Fawcett et al., 1992). However there is some evidence to suggest that LFA-1/ICAM-1 and CD2/LFA-3 may be able to augment cytotoxicity by CD8 CTLs (de Waal Malefyt et al., 1993) and so play a role in the effector rather than the initiation phase of the immune response.

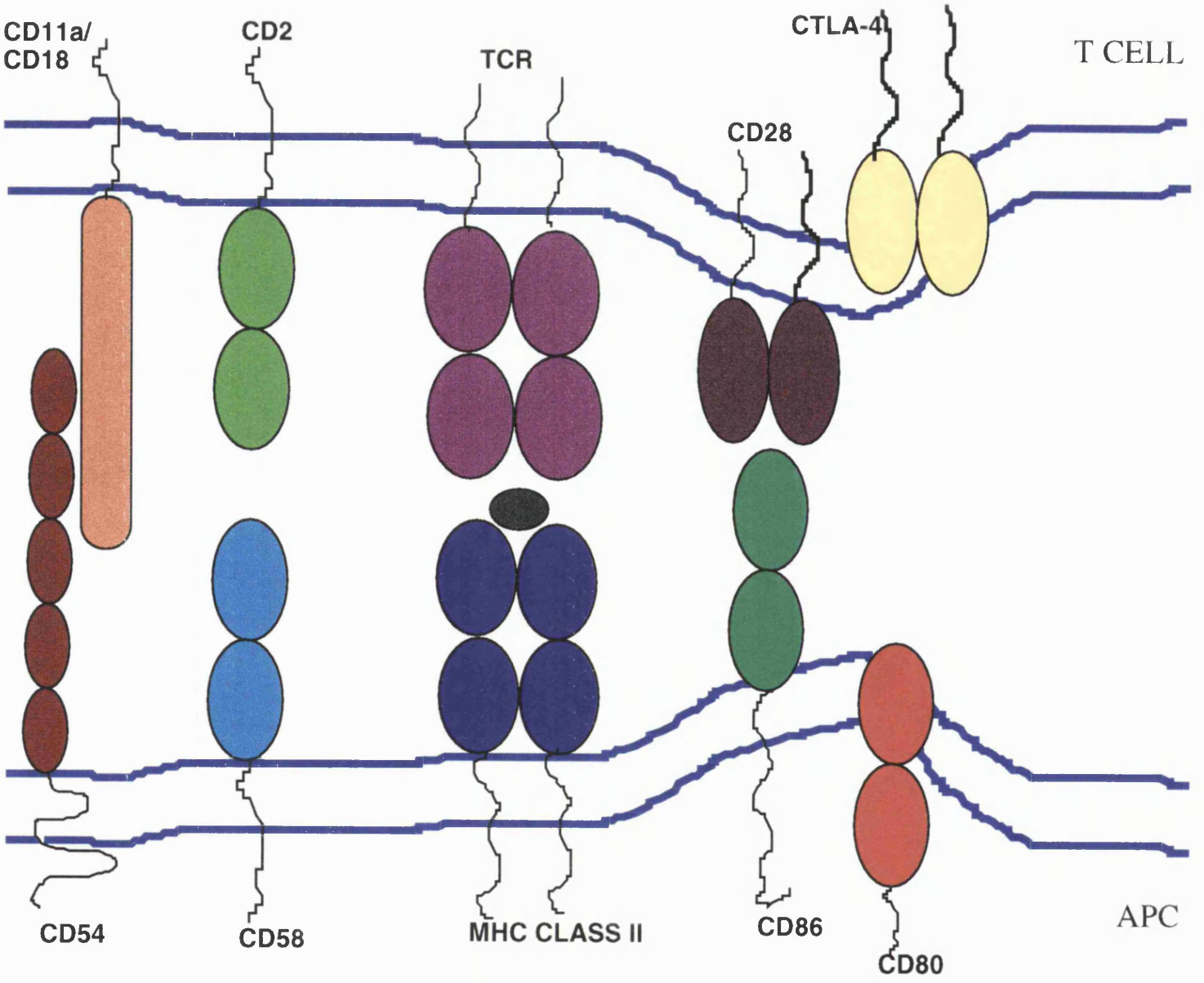


Figure 1.2 T Cell -APC Interaction

The interaction between the TCR on T cells and the MHC-antigen complex appears to involve other cell surface molecules such as CD2, LFA-3 and LFA-1 which help increase adhesion between the two cells and aid the TCR - MHC interaction. There is evidence to show that TCR only signalling in response to MHC-antigen ligation is insufficient to activate T cells and initiate T cell proliferation. This inability of T cells to respond to TCR only signalling can be beneficial to the individual as a means of controlling potentially inappropriate T cell responses. This is known as peripheral tolerance.

Peripheral tolerance

Although it is essential that the T cells circulating in the periphery are capable of detecting foreign antigen and are not self reactive, the selection process that takes place during T cell development in the thymus is not foolproof. Failure of this selection process can be seen by the presence of auto-reactive T cells in auto immune diseases and by the failure of T cells to detect cancerous cells which often express inappropriate antigens on their surface.

Because of the large number of TCRs produced by TCR gene rearrangements, it is likely that some of the TCRs will be able to recognise self antigens despite thymic selection. Mechanisms for switching off these self-reacting T cells are known as peripheral tolerance. Evidence for the existence of peripheral tolerance has been demonstrated using transgenic mice with thymic defects in negative selection during T cell development. Lack of negative selection would result in the generation of autoreactive T cells but the mice showed no overt signs of autoimmunity (Amakawa et al., 1996). However when transgenic mice with defective peripheral tolerance mechanisms were examined the mice died as a result of autoimmune syndromes (Sadlack et al., 1995, Tivol et al., 1995 and Parijs et al., 1996). Thus peripheral tolerance may be a major mechanism for controlling self-reactive T cells.

There are both *in vitro* and *in vivo* models for peripheral tolerance. Studies by Jenkins & Schwartz (1987) used an *in vitro* model of T cell tolerance to examine IL-2 driven proliferation of CD4 T cells clones in response to antigenic peptide presented by syngeneic APCs. If the APCs were pre-treated with chemical fixative prior to presentation of the antigenic peptide the T cell clones became unresponsive (anergic). This suggested that the fixed APCs, although able to present the antigenic peptide to the T cell clones, were unable

to provide all the necessary signals required for T cell activation. This would suggest that self-reactive T cells which interact with self antigen on cells which could not provide all the required stimuli for T cell activation (i.e. non professional APCs) could be tolerised. The induction of anergy in T cells given insufficient stimuli has also been demonstrated using anti-CD3 mAbs only to stimulate T cells. The T cells were found to be insensitive to restimulation (Wolf et al., 1994).

In vivo evidence for peripheral tolerance has been demonstrated in mice (Ramensee et al., 1989). Most murine T cells that express the Vβ6 TCR gene segment are reactive against the Mls-1a antigen. Mls-1b mice were made unresponsive to Mls-1a antigens following intravenous immunisations of the Mls-1a antigen. It was thought that as the Mls-1a antigen was found on resting B cells in the Mls -1b mice, the Mls-Ia antigen was presented to the T cells by the B cells , a non- professional APC. The unresponsiveness of the Mls-1b mice was not due to clonal deletion of T cells expressing Vβ6 TCRs but was due to anergy of the normally reactive Vβ6 TCR expressing T cells. The lack of sufficient stimulatory signals by the APCs resulting in peripheral tolerance of T cells suggests that T cell interactions with APCs require additional stimuli to those produced by TCR-MHC interactions.

Induction of anergy in T cells by TCR only stimulation is not the only possible outcome of insufficient stimulation of the T cells. Clonal deletion is also possible following TCR only stimulation. *In vitro* studies using superantigens such as staphylococcal entertoxin B (SEB) have shown that clonal deletion of SEB- specific murine thymocytes and murine peripheral T cells occurred in response to stimulation by SEB (D'Adamio et al., 1993). However not all the SEB specific T cells were deleted and those remaining SEB-specific T cells could proliferate in response to SEB. Human thymocytes and peripheral T cells have also been shown to undergo activation induced cell death (apoptosis) when stimulated through the TCR using SEB in the absence of accessory cells (Groux et al., 1993). Apoptosis has also been demonstrated in T cell lines using superantigens (Damle et al., 1993). This activation induced cell death could be inhibited by the use of mAbs to LFA-1. The T cells used in this case had already been primed with antigen and the induction of apoptosis occurred when the cells were restimulated with superantigens. This suggests that this may be a mechanism for preventing repeated stimulation of T cells and allowing an immune response to subside. The blocking of apoptosis using mAbs to LFA-1 suggests a role for LFA-1 in the control of the immune

response. *In vivo* data using mice have also shown that superantigens are able to induce apoptosis (Wahl et al., 1993). As in the *in vitro* experiments, the initial clonal deletion of T cells was followed by clonal expansion of the remaining superantigen - specific T cells.

The results of all these experiments suggest that TCR interaction with MHC-antigen complexes on APCs can induce three possible outcomes - T cells are rendered unresponsive, the T cells undergo programmed cell death (apoptosis) or T cells can be activated if sufficient signals are supplied i.e. the T cells are costimulated.

CD28 and T cell costimulation

Peripheral tolerance is a way of ensuring that self -reactive T cells can be inactivated and the potentially deleterious consequence of their activation is prevented. However peripheral tolerance also gives an insight into the signals required for T cell activation. TCR only signalling has been shown to result in either anergy or programmed cell death for the T cells. This suggests that for T cells to be activated additional signals are required. The induction of anergy by using chemically fixed APCs demonstrated by Jenkins & Schwartz (1987) suggested that the APCs could no longer provide all the signals required for T cell activation even though they could still present antigen to the T cells. Without costimulatory signals the T cells were not activated and as a consequence the T cells were unable to clonally expand and generate the signals required to activate the other components of the immune response. As induction of T cell proliferation is a key feature of the initiation of an immune response, the costimulatory signals required to induce T cell activation are a critical component of the initiation phase of an immune response.

Although other surface molecules (e.g. CD2, CD58) have been shown to provide help to T cells by increasing adhesion between the T cells and the APCs they are unable to provide the necessary costimulatory signals for T cell activation. However there is evidence that CD28 which is expressed on the T cell surface is able to provide the necessary costimulatory signals for T cell activation. Before examining the evidence for CD28 involvement in T cell activation, a review of the data known about CD28 and its ligands will be given.

The CD28 family of receptors

There are two known members of the CD28 " family" of receptors - CD28 and CTLA-4. Both receptors have been cloned and analysed in some detail at both the DNA and protein level. The genes for both receptors are closely linked on human chromosome 2 (2q33-34) and share the same genomic organisation, suggesting a common evolutionary origin (Harper et al., 1991). However there is only 30% homology between the genes.

CD28 is a homodimer composed of two glycosylated 44kDa chains and is a member of the immunoglobulin superfamily. Each chain contains a single disulphide - linked extracellular "Ig - like" domain linked via transmembrane region to a short cytoplasmic tail of 41 amino acids (figure 1.3) (Aruffo and Seed 1987). This cytoplasmic domain is thought to be responsible for generating costimulatory signals.

CTLA-4 is also a disulphide - linked homodimer comprising two glycosylated 20kDa chains. The single disulphide- linked extracellular domain is linked via a short amino acid stretch to a transmembrane region and short cytoplasmic domain of 36 amino acids (figure 1.3) (Linsley et al., 1991b). Although there is 100% conservation between the cytoplasmic domains of mouse and human CTLA-4 , suggesting a strongly conserved function, homology between human CD28 and CTLA-4 is as low as 30% (Harper et al., 1991). This suggests the possibility that the signals generated by these two receptors are different and may produce very different responses.

Expression of the two proteins on T cells is markedly different. CD28 is expressed at low levels on CD4+CD8+ thymocytes, with increasing expression occurring as the thymocytes mature. Peripheral T cells have similar levels of expression to the maturing thymocytes with 95% of $CD4^+$ and 50% of $CD8^+$ T cells expressing high levels of CD28 on their surface. Unlike CD28, CTLA-4 is not constitutively expressed and is only expressed on T cells after they have been activated via TCR/CD3 and CD28 ligation. The level of CTLA-4 expression on the T cells is only 1-2% of that of CD28 (Freeman et al., 1992; Linsley et al., 1992a).

Although CD28 and CTLA-4 are members of the same "family" of receptors there are a number of differences - both in their homology and their

expression. Despite these differences they have both been shown to bind the same ligands.

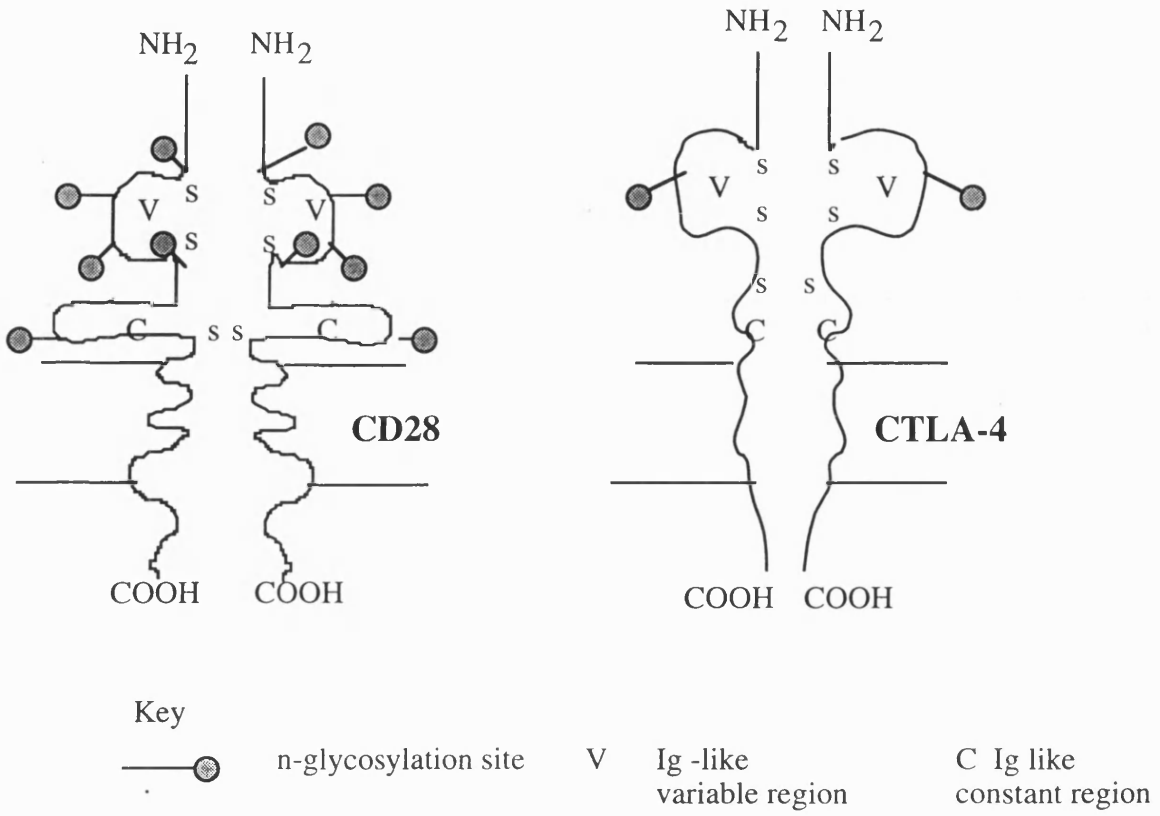


Figure 1.3 CD28 receptor family

The B7 family of ligands.

CD28 was shown to play an important role in T cell activation as long ago as the 1980s. However it was not until 1990 that a natural ligand for CD28 was discovered (Linsley et al., 1990).

B7, a B cell activation antigen (designated CD80 at the 5th International Workshop on Human Leukocyte Differentiation Antigens) was found to bind to CD28 and mediate adhesion between B cells and T cells (Linsley et al., 1990). In 1993, a second ligand was discovered by two groups of researchers working independently. B7-2 (originally known as GL1) was discovered using mAbs generated in rats immunised with activated mouse B cells and was distinct from CD80 (Hathcock et al., 1993; Freeman et al., 1993). Using a monoclonal antibody that blocked lymphocyte costimulatory signals but did

not bind to CD80, the second ligand, B70, was discovered which could bind to both CD28 and CTLA-4 (Azuma et al., 1993a).

The genes for B7-2 and B70 have been isolated and comparison of their nucleotide sequence reveals they are the same gene. However the published protein sequences of B7-2 and B70 show an additional six amino acids at the amino terminal of the B7-2 sequence which may be due to an alternative initiation site. B7-2 and B70 are considered to be the same and the second ligand for CD28 (Azuma et al., 1993a, Freeman et al., 1993). They were designated CD86 at the 5th International Workshop on Human Leukocyte Differentiation Antigens.

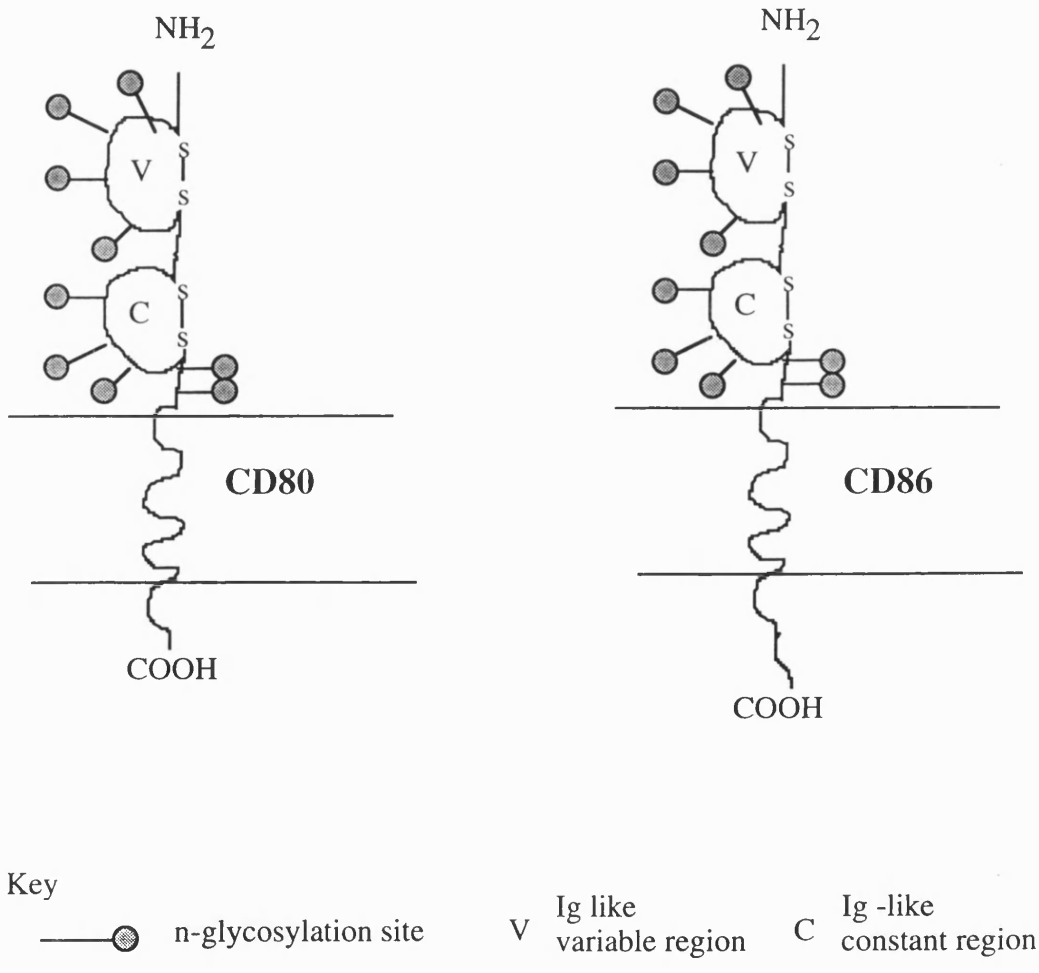


Figure 1.4 Schematic representation of B7 family of ligands showing similar overall domain structure. CD86 has an extended cytoplasmic domain which may be involved in signalling (Freeman et al., 1989, Azuma et al., 1993a, Freeman et al., 1993).

Both CD80 and CD86 are members of the Ig gene superfamily and comprise a single Ig V- like and single Ig C2- like extracellular domain (Figure 1.4) (Freeman et al., 1989, Azuma et al., 1993, Freeman et al., 1993). Comparison of the predicted amino acid sequences shows only 25% homology between CD80 and CD86. If homology between mouse and human CD80 and between mouse and human CD86 is examined, overall homology is much higher at 45% and 50%. This homology is concentrated in the extracellular domains of the proteins with their cytoplasmic sequences showing a much reduced homology (23% and 10% respectively). There are differences in the cytoplasmic domains with CD80 having a short 19 amino acid cytoplasmic tail whereas CD86 has an extended cytoplasmic domain which contains phosphorylation sites for protein kinase C (Azuma et al., 1993, Freeman et al., 1993). This would suggest that CD86 but not CD80 may have a signalling function in the APCs it is expressed on.

Like their counter-receptors CD28 and CTLA-4, there are differences in the surface expression of CD80 and CD86. Although both CD80 and CD86 are expressed on activated monocytes, activated B and T cells, CD86 is also expressed on resting monocytes. In addition, CD86 expression is upregulated early during B cell activation (expressed after 6 hours with maximal expression 24 hours) whereas CD80 is not detected until much later (24 hours after activation and reaches maximal levels after 48 hours). There is also a difference in the duration of expression of CD80 and CD86 on activated B cells with levels of CD86 remaining high 96 hours after stimulation whereas expression of CD80 has rapidly declined by that time (Hathcock et al., 1994). In addition not all activation signals resulted in upregulation of CD80 expression on B cells e.g. anti-Ig stimulation of B cells resulted in upregulation of CD86 but not CD80 (Lenschow et al., 1994). These results suggest that CD80 and CD86 expression may be regulated independently.

An examination of the receptor binding characteristics of both CD80 and CD86 (Linsley et al., 1994) shows that both bind CTLA-4 with 100 fold higher affinity than they bind CD28. The binding site on CD28 and CTLA-4 for CD80 is a conserved hexapeptide motif in the complementarity determining region 3 (CDR3). However, the greater binding characteristics of CTLA-4 has been shown to be due to non - conserved residues in the CDR-1 and CDR-3 regions (Peach et al., 1994). Although both ligands bind CD28, CD86 has slightly lower (2 - 3 -fold) overall receptor binding properties. In contrast, binding to a mutant form of CTLA-4 (mutated in the hexapeptide

binding motif) showed a significant difference with CD80 binding being >200 - fold better than CD86. This suggests that CD80 and CD86 may interact differently with the CDR-3 region of CTLA-4. Both CD80 and CD86 have similar binding kinetics but their rates of dissociation are markedly different with CD86 dissociating from CTLA-4-Ig 5 - 8 fold faster than CD80 (Linsley et al., 1994). Although there are differences in both the binding determinants used by CD80 and CD86 and the kinetics of their association with CD28 and CTLA-4 their ability to maintain similar binding avidity to their ligands suggests that they are not redundant molecules but have distinct functions.

CD28 costimulation of T cells.

Initial studies in the 1980s showed that ligation of CD28 using anti-CD28 mAbs greatly enhanced T cell proliferation when T cells were stimulated with polyclonal T cell activators such as phorbol myristate acetate (PMA), phytohaemagglutinin (PHA), anti-CD3 and anti-CD2 mAbs (reviewed by June et al., 1990). This suggested that CD28 could provide costimulatory signals which would induce T cell activation. More recent *in vitro* studies have examined the effect of lack of CD28 costimulation on T cell activation. Human tetanus-toxoid specific clones were rendered non-responsive to secondary stimulation by costimulation-competent APCs if initially activated by CD80-deficient APCs (Gimmi CD et al., 1993). Similarly interruption of CD28 -CD80 signalling by anti-CD28 Fab fragments or the chimeric molecule CTLA-4-Ig (comprising the extracellular domain of CTLA-4 which includes the CD80 binding site fused to the constant region of an IgG antibody) resulted in peripheral blood T cells being hyporesponsive to secondary stimulation by specific alloantigens (Tan et al., 1993).

This important role of CD28 has also been demonstrated *in vivo* using knockout mice. In CD28 "knockout" mice, although T cell development and negative selection occurred normally, the animal's ability to produce IL-2 in response to mitogenic stimulation was diminished (Shahinian et al., 1993). Antibody production in these mice was also reduced. However, T cell responses were not totally absent as CTLs were able to respond to lymphochoriomeningitis virus (LCMV). This response may be due to the CTLs being mediated by cytokines other than IL-2 and not regulated by CD28 (Shahinian et al., 1993). Further studies with CD28-deficient mice have shown that T cell responses to staphylococcal enterotoxins B were impaired (Mittrucker et al., 1996) and that the presence of CTLA-4 (a counter receptor

to CD80) on CD28-deficient mice T cells did not result in T cell activation when costimulated by CD80 transfectants indicating that CTLA-4 ligation was not costimulatory.

Not only has lack of CD28 costimulation been shown to induce unresponsiveness in T cells but it has also be demonstrated that CD28 ligation can in fact reverse the induction of antigen - specific unresponsiveness. $Th1$ T cells encountering antigen - MHC complexes in the absence of costimulation were rendered anergic and were unresponsive to restimulation. However this anergic state could be reversed by the addition of exogenous IL-2 or more importantly by ligation of CD28 during the initial stimulation of the T cells (Jenkins et al., 1991; Harding et al., 1992). The necessity for CD28 ligation to overcome T cell anergy helps to explain the lack of T cell stimulation in CD28 deficient mice despite the presence of CTLA-4 (which can also bind CD28 ligands).

The evidence from both *in vitro* and *in vivo* studies demonstrate that lack of costimulation through CD28 resulted in T cell hyporesponsiveness. When these data are coupled with the mAb data showing enhanced T cell proliferation using anti-CD28 mAbs in conjunction with mitogens or anti-CD3 and CD2 mAbs, this suggests an essential requirement for CD28 costimulation in T cell activation.

With the discovery of the natural ligand for CD28 (CD80) it was possible to examine the effect of ligand binding to CD28 on T cell activation. CD28 expressed on CHO cells was shown to bind to lymphoblastoid and leukaemic B cell lines. This adhesive function was CD80 specific as it could be inhibited by anti-CD80 mAbs (Linsley et al., 1990). Further research demonstrated that CD80 could costimulate T cells in conjunction with anti - CD3 mAbs resulting in T cell proliferation and IL-2 secretion. This interaction was also shown to be CD80 specific as it could be abrogated with anti- CD80 mAb (Gimmi et al., 1991; Linsley et al., 1991a). Although CD80 was able to bind to CD28 and initiate T cell activation, it was observed that addition of the chimeric molecule CTLA4-Ig could abrogate CD28 - CD80 costimulation of T cells (Linsley et al., 1991b). As CD80 has a higher affinity for CTLA-4 than CD28, preferential binding of CD80 to CTLA-4 would prevent the essential costimulation of the T cells by CD28 resulting in abrogation of T cell proliferation. CD80 ligation of CD28 has also been shown to provide the costimulatory signal required for alloactivation of CD4 T cells (Koulova et al.,

1991). Alloactivation required both TCR binding and CD28 costimulation as mAbs to CD3, MHC Class II, CD28 and CD80 could inhibit T cell proliferation. All these experiments demonstrated that T cell activation required two signals - one generated via the TCR and the second through ligation of CD28.

The generation of the chimeric protein CTLA4-Ig meant that the role of CD28 - CD80 costimulation could be examined indirectly *in vivo*. Treatment of mice with CTLA4-Ig resulted in long term acceptance of pancreatic islet cell xenografts (Lenschow et al., 1992) and the inhibition of T cell dependant antibody responses (Linsley et al., 1992b). The results of the xenograft experiments suggests an essential role for CD28 in the generation of an immune response, in this case, to foreign tissue. The inhibition of antibody responses suggests that CD28 may also be involved in the later immune events such as T cell dependant B cell responses. Further *in vivo* studies using transgenic mice (Harlan et al., 1994) demonstrated directly that costimulation via CD28 was required to activate T cells, resulting in autoimmunity in this mouse model of diabetes. Transgenic mice were produced which expressed CD80 on their pancreatic beta cells. Contact with these cells did not result in the non-specific activation of T cells. Similarly transgenic mice produced which expressed both the lymphocytic choriomeningitis virus glycoprotein (GP) via H-2D^b and the TCR specific for H-2D^b presented GP. This resulted in the production of CD8 self - reactive T cells but these cells were not activated as shown by the lack of spontaneous lymphocyte infiltration or diabetes. However cross breeding of the mice to produce triple transgenic mice i.e. expressing the self antigen, a TCR specific to that antigen and the costimulatory ligand CD80 resulted in the mice developing diabetes due to immune-mediated beta cell destruction. These results clearly show that both TCR specific responses and costimulation via CD28 are required for T cell activation.

Research into how CD28 costimulatory signalling can induce T cell activation has demonstrated a number of different effects. CD28/CD80 interactions have been shown to provide co-stimulatory signalling in an antigen specific manner resulting in IL-2 production (Linsley et al., 1991a; Jenkins et al., 1991). IL-2 is the major T cell growth factor required for T cell proliferation. It exerts its effect on T cells by binding to a specific receptor - the IL-2 receptor (IL-2R) which induces intracellular signalling eventually resulting in T cell proliferation (Minami et al., 1993). When T cells were stimulated with PMA

in conjunction with mAbs to CD28 there was an increase in IL-2 gene transcription & mRNA stability (Lindsten et al., 1989; Fraser et al., 1991). As IL-2 production is a key feature of T cell activation and T cells proliferate in response to IL-2, the CD28 costimulatory signal would appear to enhance IL-2 production in two ways - increasing gene transcription and increasing stability of the transcripts allowing more translation of the IL-2 mRNA resulting in increased IL-2 production.

Another result of CD28 costimulatory signals is an increase in IL-2R expression. The IL-2R consists of three distinct subunits - the α chain, the β chain and the γ chain. Only the γ chain is expressed constitutively in all T cells, with the β chain being found constitutively expressed on CD8 T cells but not CD4 T cells. Expression of different combinations of the three subunits (α, β and γ chains) gives rise to various forms of IL-2R each with different binding affinities for IL-2 with the highest affinity receptor comprising all three subunits (reviewed by Minami et al.,1993). Binding of IL-2 to its receptor induces intracellular signalling eventually resulting in T cell proliferation. However for signal transduction to occur both the β and γ chain must be present. CD28 costimulation has been shown to up-regulate long-term IL-2Rβ chain expression (Cerdan et al., 1995). This upregulation of IL-2R is similar to the increased IL-2 production induced by CD28 costimulation as it is achieved by increasing IL-2Rβ gene transcription and increasing IL-2R mRNA stability. As the γ chain of the IL-2R is constitutively expressed on T cells and both the γ and β chains are required for IL-2R signalling, induction of the β chain of the IL-2R would ensure that the T cells expressed an IL-2R capable of both binding IL-2 and generating the necessary intracellular signals resulting in T cell proliferation. As CD28 also increases IL-2 production CD28 costimulation ensures that both the cytokine and its receptor are present during T cell activation so enabling T cell proliferation in response to IL-2 binding to the IL-2R to occur.

This evidence seems to suggest that the role of CD28 costimulation is to induce IL-2 and IL-2R expression and so promote T cell proliferation in response to the IL-2. *In vivo* studies with CD28 knockout mice demonstrated that although T cell development and negative selection occurred normally, the animal's ability to produce IL-2 in response to mitogenic stimulation was diminished (Shahinian et al., 1993). This seems to confirm the CD28 costimulatory role as being enhancement of IL-2 production. However this may not be the only function of CD28 costimulation. Evidence from CD28

deficient mice that naive T cells can respond to mitogen stimulation but this response cannot be sustained (Lucas et al., 1995) suggests that CD28 signalling plays a role in T cell survival. The evidence from peripheral tolerance suggests that insufficient T cell signalling due to lack of costimulatory signals can result in T cell anergy or activation induced cell death. CD28 costimulation can prevent both of these outcomes. By providing the essential costimulatory signals for T cell activation CD28 prevents anergy induction which may occur in response to TCR only signalling. Another consequence of CD28 signalling is the increased transcription of the bcl -xl gene (Boise et al., 1995) which is known to protect cells from apoptosis. CD28 signalling is therefore essential to prevent TCR only signalling inducing anergy or apoptosis in T cells and so ensuring survival of the T cells.

CTLA-4 and T cell costimulation.

The discovery of CTLA-4 (Brunet et al., 1987) with its structural homology to CD28 suggested that there were perhaps two T cell receptors capable of activating T cells. Initial studies have demonstrated that CTLA-4 could not provide the necessary costimulatory signals for T cell activation but in conjunction with CD28 signalling could synergistically augment TCR-induced proliferation of pre-activated T cells (Linsley et al., 1992a). The inability of CTLA-4 to provide costimulatory signals to T cells was also suggested by generating CD8/CD28 and CD8/CTLA-4 chimeric molecules (the cytoplasmic domain of CD28 or CTLA-4 is fused to CD8) and transfecting them into T cells. Crosslinking of the CD8/CD28 construct generated costimulatory signals whereas crosslinking of the CD8/CTLA-4 construct did not (Stein et al., 1994). The results of this experiment demonstrated that the signals generated through the CTLA-4 cytoplasmic domain were not costimulatory. Since the homology between the cytoplasmic domains of CD28 and CTLA-4 is only 30% it is unlikely that the signals generated through these cytoplasmic domains would be identical. However the original experiments showing augmentation of CD28 costimulation of T cells suggested that the function of CTLA-4 ligation may be to enhance proliferation and prolong IL-2 production and T cell proliferation (Linsley et al., 1992a).

Further insights into CTLA-4 function were generated by research on murine T cells which demonstrated that anti-CTLA-4 mAbs and its Fab fragments were able to augment T cell responses (Walunas et al., 1994). This would

appear to confirm the idea that CTLA-4 can augment T cell proliferation. However if the mAbs were cross-linked the anti-CTLA-4 mAbs appeared to inhibit T cell responses. It would appear from the results of the crosslinking experiments that the apparent augmentation of T cell proliferation by CTLA-4 was possibly due to the anti-CTLA-4 mAbs and Fab fragments blocking CTLA-4 inhibitory signals. This has to some extent been confirmed by blocking CTLA-4 signals using anti-CTLA-4 mAbs which has resulted in increased IL-2 production and T cell proliferation in T cells stimulated by anti-CD3 and anti-CD28 mAbs (Krummel and Allison, 1995; Krummel and Allison, 1996). Compelling evidence of an inhibitory role for CTLA-4 has been shown *in vivo* in CTLA-4 knockout mice. These animals suffer from a lymphoproliferative disorder due to the presence of activated T cells which infiltrate a number of major organs resulting in early death of the animals (Waterhouse et al., 1995).

Recent *in vitro* research (Krummel and Allison 1996, Walunas et al., 1996) has demonstrated that CTLA-4 inhibits T cell proliferation by blocking IL-2 production and IL-2R upregulation. This suggests that CTLA-4 signalling opposes the costimulatory effects of CD28 which result in IL-2 production, IL-2R upregulation and T cell proliferation. As CD28 signalling has also been shown to enhance T cell survival by inducing transcription of the protective gene bcl-$_{xl}$, it is possible that CTLA-4 signalling may affect T cell survival. However research into the inhibitory role of CTLA-4 on T cell activation has shown that CTLA-4 inhibited IL-2 production and T cell proliferation in murine T cells stimulated with anti-CD3 and anti-CD28 mAbs in the absence of cell death (Krummel and Allison, 1996). Further research has shown that the inhibition of T cell proliferation by CTLA-4 is due to blocking cell cycle progression by arresting the cell cycle in the G_1/S phase (Krummel and Allison, 1996; Walunas et al., 1996). The ability of CTLA-4 to inhibit T cell proliferation was more pronounced at later time points (72 hours) following the initial activation of the T cells. This is in line with the data that CTLA-4 surface expression relies on CD28-dependent costimulation and peak surface expression of CTLA-4 occurs 48-72 hours following T cell activation (Walunas et al., 1994).

Ligation of CD28 by either CD80 or CD86 has been shown to be essential for T cell activation resulting in IL-2 production and T cell proliferation. In contrast CTLA- 4 ligation produces the opposite signals i.e. inhibition of IL-2 production and T cell proliferation. For the effects of ligation of CD28 and

CTLA-4 to be achieved intracellular signals must be generated within T cells following ligation of these surface receptors. Intracellular signalling in T cells is a highly complex mechanism which is still being elucidated. However some information about the signalling pathways used following TCR and CD28 stimulation is available.

Intracellular signalling mechanisms.

The recognition of foreign antigen by the TCR/CD3 complex and the provision of costimulation through CD28 results in the activation of the T cell. This generates a cascade of events including the production of cytokines, T cell proliferation, the activation of B cells and phagocytic cells, antibody production and the eventual destruction of the foreign antigen. However for this outcome to occur the recognition of antigen and the ligation of costimulatory molecules on the surface of the T cell must generate intracellular signals which will eventually result in the transcription of the appropriate genes to generate the cellular response.

Intracellular signalling can be stimulated by a variety of extracellular stimuli e.g. ligand binding to surface receptors, mitogens, hormones and cytokines. The signalling pathways generated will produce a variety of responses depending on the initial stimulus and the cell involved. However studies have shown that there are groups of similar proteins which are involved in intracellular signalling. One of these groups is that of protein tyrosine kinases (PTKs). These enzymes are able to phosphorylate the tyrosine amino acids of other proteins which then changes the conformational shape of the molecule and if the protein is an enzyme may cause a change in its activity (Ullrich and Schlessinger 1990). The protein targets of PTKs often contain a conserved protein module of approximately 100 amino acids known as the Src homology 2 (SH2) domain (Pawson and Gish 1992). Proteins with SH2 domains often possess a distinct region of approximately 50 amino acids, the Src homology 3 (SH3) domain which is thought to regulate protein - protein interactions (Clark et al., 1992). For binding of a protein target to a PTK to occur a specific residue in the PTK must be phosphorylated. However it is the amino acid sequence surrounding the phosphorylated amino acid which determines which protein target will bind (Cantley et al., 1991). The protein target may itself be a PTK as many SH2/SH3 containing proteins have intrinsic PTK activity. As some receptors do not contain intrinsic kinase activity it may be possible for them to recruit intracellular PTKs by phosphorylation of a tyrosine residue in

their cytoplasmic tail which will then interact with the SH2 domain of a PTK so beginning the biochemical signalling pathway. The eventual effect of many signalling pathways is the induction of transcription factors which travel to the nucleus and bind to the promoter region of a specific gene so inducing gene transcription which will eventually result in the required cellular outcome e.g. cytokine production.

TCR/CD3 complex signalling.

The interaction of the TCR/CD3 complex with the MHC/antigen complex on the APC results in the generation of a variety of intracellular signals in the T cell. The α and β chains of the TCR have short cytoplasmic domains of approximately 5 amino acids and are not involved in signal transduction. However the cytoplasmic domains of CD3 are longer (40-113 amino acids) and contain copies of a tyrosine based activation motif (TAM), a conserved domain of precisely spaced amino acids found in other signal transduction chains such as the B cell receptor (Reith, 1989). Within the TAM are two tyrosine residues separated by ten amino acids which are potential sites for phosphorylation (Sancho et al., 1993). Multiple copies of the TAM are found in the ζ chain and a single copy is found in γ and δ chains with a partial copy in the ε chain of CD3. This may allow the TCR to couple to divergent signalling pathways.

As the TCR/CD3 complex has no intrinsic protein tyrosine kinase activity and a number of cellular proteins are tyrosine phosphorylated as a result of TCR stimulation, cytoplasmic PTKs must be associated with proximal TCR signalling. Two PTKs have been shown to associate with the ζ chain of the CD3 complex. P59 ^{fyn} has been co-immunoprecipitated with the ζ chain of CD3 (Samelson et al., 1990) and another PTK Zap-70 has also been shown to associate with the ζ chain of CD3 (Chan et al., 1992). The association of Zap-70 with the TCR/CD3 complex results in Zap-70 itself being tyrosine phosphorylated. It is thought that phosphorylation of the ζ chain of CD3 possibly by P59 ^{fyn} results in recruitment of Zap-70 via one or both of the two SH2-like domains of Zap-70 through an SH-2/phosphotyrosine association (Koch et al., 1991). More recent studies have shown that the tandem SH2 domains of ZAP-70 bind distinct tyrosine phosphorylated TAMs of the TCR/CD3 complex (Isakov et al., 1995). The substrates of these PTKs are unknown but p59 ^{fyn}, being a src-like kinase, contains an SH2 domain

which may bind other phosphotyrosine containing proteins (possibly other PTKs). Research into the role of p59 ^{fyn} in mouse T cells suggests that the PTK may be involved in TCR induced calcium mobilisation, although the exact mechanism is unknown (Stein et al., 1992). More recent research using human Jurkat T cells suggests that p59 ^{fyn} can regulate calcium influx but the mechanism is distinct from phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis.

A third PTK is thought to be indirectly associated with the TCR via the co-receptors CD4 or CD8. Although TCR/CD3 interactions with MHC-antigen complexes are considered to provide the first signal required for T cell activation, the surface receptors CD4 or CD8 expressed on T cells are known to help stabilise TCR/CD3 interactions with MHC-antigen complexes (Norment et al., 1988). The cytoplasmic domains of CD4 and CD8 associate non-covalently with the PTK p56^{lck} through cysteine-dependant interactions (Shaw et al., 1989) and the association of p56^{lck} with CD4 and CD8 suggests a potential role in signal transduction for these TCR co-receptors. Evidence for the requirement of p56^{lck} activity in T cells comes from a number of studies of mutant cell lines. A CD4-dependent antigen-specific murine T cell lacking endogenous CD4 could only demonstrate TCR function after the introduction of CD4 molecules which could associate with p56^{lck}. Mutant CD4 molecules that were unable to associate with p56^{lck} were unable to restore TCR function (Glaichenhaus et al., 1991). When two p56^{lck} deficient T cell lines (a Jurkat human T cell leukaemia line and a murine cytotoxic T cell clone) were examined both were found to be defective in TCR-mediated signalling. Responsiveness to TCR cross-linking could be restored by transfection with lck expression plasmids (Karnitz et al., 1992, Straus et al., 1992).

CD45 which is expressed on all T cells may also play a role in TCR signalling. From the cDNA of CD45, 8 possible isoforms can be generated by differential usage of the 3 exons which encode the extracellular domain of CD45 (Streuli et al., 1987). However 2 isoforms predominate on T cells - CD45RA which is found on naive T cells and CD45 RO which is present on memory T cells (Akbar et al., 1988). The cytoplasmic tail of CD45 has been shown to contain phosphotyrosine phosphatase (PTP) activity (Tonks et al., 1988) and evidence for CD45 involvement in T cell signalling has been demonstrated using mutant CD45$^-$ mouse T cell clones and a CD45$^-$ T -leukaemia cell lines both of which were unresponsive to signalling via the TCR (Pingel and Thomas, 1989; Koretzky et al., 1990). CD45 can dephosphorylate the regulatory

carboxy-terminal tyrosine residue in the kinase domain of the protein tyrosine kinases (PTKs) $p56^{lck}$ and $p59^{fyn}$ resulting in increased kinase activity (Mustelin et al., 1989; Mustelin et al., 1992) and as already seen both these PTKs are associated with the TCR.

Stimulation of the TCR results in phosphorylation of a variety of cellular proteins including the ζ subunit of CD3 and phospholipase C γ1 (PLC γ1). The phosphorylation of PLC-γ1 increases its catalytic activity resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These secondary messengers raise intracellular calcium levels and stimulate protein kinase C (PKC) in a calcium dependent manner, respectively. The increased calcium levels result in the activation of calcineurin, a calcium/calmodulin dependent protein serine/threonine phosphatase whose target is unknown but may be the cytosolic component of a transcription factor (NF-AT) involved in IL-2 gene regulation. Activation of PKC by DAG results in the activation of a number of other kinases including MAP kinase whose phosphorylation targets are a number of transcription factors which may be involved in lymphokine gene expression. PKC activation also results in transcription of a number of other genes such as c-fos and c-jun which form part of the transcription factors (AP-1 and NF-AT) involved in IL-2 gene regulation as well as c-myc which is involved in cell division (reviewed by Fraser et al., 1993; Perlmutter et al., 1993).

Although TCR ligation initiated this complex network of interacting signal transduction pathways, the signals generated are insufficient for T cell proliferation. As has already been discussed, TCR signalling in the absence of other important signals may result in functional inactivation or clonal anergy (Jenkins and Schwartz, 1987) or to activation-induced T cell death or apoptosis (Groux et al., 1993; Damle et al., 1993) by upregulation of the ligand for the death receptor CD95 (Fas) on T cells (Dhein et al., 1995). The requirement for costimulatory signals through CD28 has already been shown. As with TCR-mediated intracellular signalling pathways, the intracellular signals generated following CD28 ligation are still being elucidated.

CD28 Intracellular signalling.

Like the TCR/CD3 complex, CD28 is unable to initiate intracellular signals directly as the cytoplasmic domain of CD28 has no intrinsic enzymatic activity. However like the TCR/CD3 complex the cytoplasmic domain of CD28 contains a number of consensus sequences. One of these sequences, YMNM, is similar to the core phosphotyrosine sequence which has specificity for the SH2 domain of signalling proteins. There are also two proline rich motifs which conform to SH3 binding consensus sequences. This suggests that CD28 should be able to recruit PTKs through binding via these consensus sequences. Evidence for PTK activity associated with CD28 stimulation has been demonstrated by both the tyrosine phosphorylation of a number of substrates as well as tyrosine phosphorylation of the cytoplasmic domain of CD28 itself following ligation of CD28 (Lu et al., 1992; Vandenberghe et al., 1992; August and Dupont, 1994; Pages et al., 1994). However data from studies using herbimycin A, a src-family PTK inhibitor, have suggested that CD28 may associate with more than one PTK. When CD28 was ligated using cross-linked anti-CD28 mAbs herbimycin A was able to inhibit tyrosine phosphorylation of a number of substrates and IL-2 production as well as inhibit intracellular calcium levels (Vandenberghe et al., 1992; Ueda et al., 1994). However if CD80 was used to stimulate CD28 signalling, herbimycin A had no effect on intracellular calcium levels (reviewed by Sansom et al., 1997). This suggests that CD28 can associate with both herbimycin sensitive and insensitive PTKs.

As the PTKs $p59^{fyn}$ or $p56^{lck}$ are known to associate with the cytoplasmic domains of the TCR/CD3 complex and its co-receptors CD4 or CD8, it is possible that the same PTKs may be coupled to CD28 during CD28 signalling. It has been demonstrated that CD28 crosslinking results in increased tyrosine phosphorylation of PLC γ1 and increased activity by $p56^{lck}$ and $p59^{fyn}$ (Ledbetter and Linsley, 1992) and studies using mAb ligation of CD28 have implied an association of CD28 with both $p56^{lck}$ and $p59^{fyn}$ (Hutchcroft and Bierer, 1994). This suggests that TCR and CD28 signalling may be linked possibly by sharing of PTKs.

CD80 co-stimulation of T cells has been shown to be more efficient if the CD80 and the TCR ligand are on the same cell (Liu and Janeway Jr, 1992). However another study (Sansom et al., 1993) has shown that CD80 transfected CHO cells can act as third party co-stimulators of T cells. The difference in the

results obtained in these two studies suggests that as third party costimulation can occur there is no necessity for coassociation of CD28 with the TCR. However if TCR and CD28 ligands are present on the same cell the increased efficiency may be due to increased adhesion between the T cell and APC rather than linkage of intracellular signalling pathways.

As has already been discussed CD28 ligation by CD80 (as determined by intracellular calcium levels) is insensitive to herbimycin A treatment suggesting that herbimycin-insensitive PTKs can associate with CD28. As Zap-70 is a Syk- family PTK known to associate with the TCR, it could be a potential candidate for herbimycin A insensitive PTK association with CD28. However data from studies where CD28 and interactive components of the CD28 signalling pathway were expressed in insect cells suggest that the Syk - family PTK Zap-70 does not associate with CD28 (Raab et al., 1995). It is possible that the herbimycin-A resistant PTKs are not PTKs associated with the TCR and that CD28 association with this PTK generates a completely separate signalling pathway from the TCR. There is further evidence of a separate signalling pathway by CD28.

The cytoplasmic tail of CD28 contains the consensus motif YMNM which is similar to the phosphotyrosine containing consensus motif YXXM which has specificity for the p85 subunit of phosphatidylinositol 3- kinase (PI3-kinase). This suggests direct interaction between CD28 and PI3-kinase. PI-3 kinase is a heterodimer consisting of a 85kDa regulatory subunit containing two SH2 domains and an SH3 domain tightly associated with a 110kDa catalytic subunit. PI3-kinase has dual specificity as it is a lipid and protein serine kinase.Evidence for association of CD28 with PI3-kinase has been demonstrated using the leukaemic T cell line Jurkat. Ligation of CD28 by CD80 induced accumulation of D-3 phosphatidylinositol lipids, the products of PI3-kinase activity (Ward et al., 1993). The functional significance of PI3-kinase association with CD28 has been demonstrated in murine T cell hybridomas expressing mutations in the YMNM motif of the CD28 cytoplasmic tail which are no longer able to produce IL-2 following CD28 ligation (Pages et al., 1994). As a feature of CD28 signalling is increasing both the transcription and stability of IL-2 mRNA, the association of CD28 and PI3-kinase appears to be an important part of the CD28 signalling pathway required to achieve this.

There is evidence to suggest that like the TCR, CD28 can activate phospholipase C. However there are differences in the results obtained depending on the activation state of the T cells used and the method of ligating CD28. Monoclonal antibodies to CD28 can induce phosphorylation of PLCγ-1 in Jurkat cells in the absence of crosslinking (Nunes et al., 1993). However the mAbs must be crosslinked to achieve the same effect in resting T cells and activated T cells (Couez et al., 1994). There is evidence to suggest that CD80 does not induce PLC activation suggesting that the mAb activation of PLC may be functionally redundant (Ward et al., 1993). This is to some extent confirmed by the finding that, in contract to TCR signalling, signalling through CD28 does not require calcium mobilisation. Production of IL-2 by T cells stimulated with PMA and soluble anti-CD28 mAbs is unaffected by blocking a calcium dependent signal transduction pathway using cyclosporin A (CsA) (Crabtree, 1989; June et al., 1987). However it has been suggested that the CsA-insensitive and CsA-sensitive pathways may reflect different CD28 signalling responses depending on the activation state of the T cell. It is possible that the CsA-sensitive pathway is dominant in activated T cells whereas the CsA-insensitive pathway predominates in resting T cells (June et al., 1994).

There is also evidence for CD28 induction of $p21^{ras}$ which is associated with TCR/CD3 signalling. Once again the evidence appears to depend on how CD28 is stimulated, with mAbs to CD28 but not CD80 activating $p21^{ras}$ (reviewed by Sansom et al., 1997). As can be seen by the conflicting evidence from studies on PLC and $P21^{ras}$ activation by CD28, the signalling pathways for CD28 are still being determined and it appears from a number of studies that the signals generated depend on the activation state of the T cell and the method of ligating CD28 i.e. mAbs or natural ligands. However the importance of CD28 costimulatory signals on T cell activation cannot be underestimated. The effect of CD28 signalling in conjunction with TCR signals on cytokine production will now be examined.

Transcription factor targets

The ability of CD28 to provide an intracellular signal which augments that provided by the TCR/CD3 complex and is sufficient to induce T cell proliferation and IL-2 production has been clearly demonstrated. CD28 costimulation is known to upregulate production of a number of other

cytokines including IL-4, IL-8, IL-13 and γIFN (Thompson et al., 1989; Minty et al., 1993; Weschler et al., 1994; Seder et al., 1994). The effect of CD28 signalling on the induction of cytokine production is mediated by transcriptional effects - upregulation of transcription as well as stabilisation of cytokine mRNA (Lindsten et al., 1989; Fraser et al., 1991). However CD28 signalling alone cannot induce cytokine production, TCR signalling is also required (Figure 1.5).

Transcriptional regulation of the IL-2 gene has been investigated and requires the binding of a number of transcription factors (AP-1, NF-kB, NF-AT and OCT) to the IL-2 promoter (Fraser et al., 1993). CD28 has been shown to activate IL-2 gene transcription by regulating the gene enhancer activity by the induction of a transcription factor known as the CD28 response element or response complex (CD28RE or CD28RC), (Verweij et al., 1991; Fraser et al., 1991 respectively). CD28 signalling has also been shown to be necessary in conjunction with TCR mediated signals for the generation of AP-1 (Rincon and Flavell, 1994). However another study has shown that CD28 stimulation can cause down-regulation of AP-1 (Los et al., 1994). Furthermore a more recent study has suggested that CD28 signalling in T cell blasts can result in the generation of NF-kB and AP-1 but not NF-AT. However all three transcription factors could be induced through TCR mediated signals (Edmead et al., 1996). It is possible that the CD28 signalling pathways generated varied depending on both the activation state of the T cell used and the method of ligation of CD28 (reviewed by Sansom et al., 1997). Possibly, the CD28 pathway generated may affect which transcription factors are induced. The evidence from the research into induction of transcription factors in T cell blasts (Edmead et al., 1996) suggests that NF-AT, NF-kB and AP-1 must all be present for IL-2 production. It is possible that in unstimulated T cells TCR-mediated signals are not sufficient to generate the required transcription factors and that CD28 costimulatory signals are needed not only to generate NF-AT, NF-kB and AP-1 but that the CD28RE is essential for induction of IL-2 transcription.

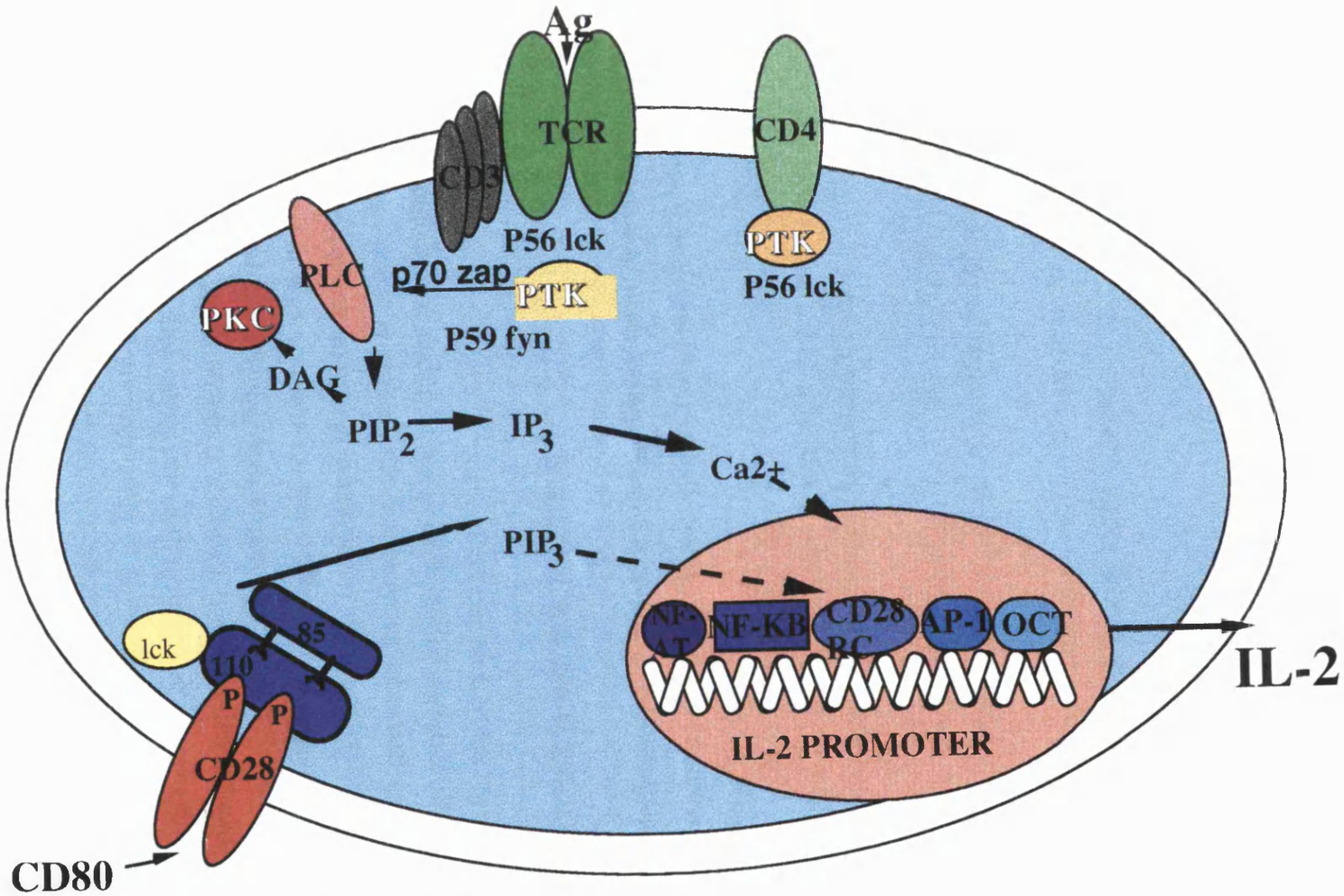


Figure 1.5 Schematic of T cell intracellular signalling.

Aims of the project

T cell activation is thought to require two signals - one through the TCR and a second costimulatory signal. It has been suggested that ligation of CD28 may provide the necessary costimulation. In the absence of CD28 costimulation T cells may become unresponsive to subsequent antigenic challenge. There are two known ligands for CD28 - CD80 and CD86 which are known to bind to CD28 with similar affinities. However it has yet to be established whether these two ligands have differential function. Whilst the importance of CD28 in activating T cells has been suggested, the role of CD28 in sustaining T cell proliferation has not been investigated. Furthermore, the mechanisms by which CD28 transmits its signalling information is poorly understood. As CD28 has no intrinsic signalling mechanism, it must recruit other intracellular molecules for signal transduction to occur.

The aims of this project were therefore to:

1. Establish assays of T cell activation which were dependant on CD28 costimulation via its natural ligands and study the kinetics with which CD28 is used in these assays,

2. Investigate the effects of CD28 engagement on previously activated T cells to compare its role in resting and pre-stimulated T cells,

3. Compare the relative abilities of CD80 and CD86 in costimulating activation in resting T cells and sustaining proliferation of activated T cells,

4. Investigate the mechanisms of CD28 signalling in normal T cells with particular relevance to the role of PI3-kinase.

CHAPTER 2

MATERIALS AND METHODS

Reagents used

The majority of standard reagents used were purchased from Sigma Chemical Co Ltd, the others are as indicated in the relevant method. A full list of suppliers including their addresses can be found in Appendix 1.

Cell culture

A variety of different cells were cultured often requiring both different culture media and different cell culture techniques. The cell culture techniques used for each cell type are detailed below and the individual cell culture media requirements are shown in Table 2.1.

Cell culture reagents and media

All the media and supplements used (except the nucleosides) were purchased from Life Technologies Ltd, the nucleosides were obtained from Sigma. Phosphate-buffered saline (PBS) tablets were purchased from Unipath Ltd. All the tissue culture plastics were obtained from Falcon Ltd.
The individual media requirements of the different cell types cultured are listed in Table 2.1 (Recipes for the culture media and additives are listed in Appendix 2).

Table 2.1 Culture media requirements of different cells used.

	CELL TYPE		
	Transfectants (CHO Medium)	CTLLS	T cell Blasts Hybridomas (Complete Medium)
Media	Dulbecco's minimal essential medium without l-glutamine (DMEM)	RPMI 1640	RPMI 1640
Foetal calf serum	10%	10%	10%
Sodium bicarbonate 7.5% (w/v)	0.4%	0.25%	0.25%
L-Glutamine	-	2mM	2mM
Sodium pyruvate	1mM	-	-
Penicillin	100 units / ml	100 units / ml	100 units / ml
Streptomycin	100 µg /ml	100 µg/ ml	100 µg/ ml
Nucleosides	26µM adenosine 28µM cytidine 29µM uridine 25µM guanosine 9µM thymidine	-	-
Additional Reagents	500ng/ml G418 - sulphate (for CD58 transfectants)	10 -15 international units (IU/ml) recombinant human Interleukin -2 (rhIL-2).	-

Cell culturing techniques

All cell culture manipulations were carried out in a Class II Biological Safety cabinet to maintain sterility and all cells were cultured in incubators at 37°C and 5% CO_2.

a) Transfectants

(Sansom et al 1993)

Chinese Hamster Ovary (CHO) K1 cells transfected with human cDNAs encoding CD58 (LFA-3), CD80 (B7) and CD86 (B70) were generated in our lab (Sansom et al 1993). The transfectants and the parental cell line were maintained in CHO Medium (see Table 2.1).

CHO cells are an adherent cell line and were regularly subcultured (normally every 2 - 3 days depending on the rate of growth of the individual transfectants). The cell culture medium was removed from the tissue culture flask. This also removed any dead cells which were no longer adherent. The adherent cells were washed with 5ml of phosphate buffered saline (PBS) which was then removed. This washing process was to ensure that all the culture medium had been removed and would have no effect on the Trypsin -EDTA used to remove the adherent cells from the tissue culture flask. 3ml Trypsin-EDTA (1 :250) (Life Technologies Ltd) was added to the culture flask which was incubated for 5 minutes at 37^{o}C. The flask was then gently tapped to aid removal of the adherent cells. The action of the Trypsin - EDTA was neutralised by the addition of 10 ml of CHO medium. The resulting cell suspension was passaged 1:10 and the cells cultured for future use. The remaining cells were either used in proliferation experiments or after examination of the surface expression of their individual human surface ligand by flow cytometry were frozen down for storage in liquid nitrogen .

b) CTLLs

(Gillis and Smith 1977).

The murine cytotoxic T cell line 2 (CTLL2) cells were obtained from the National Institute for Biological Standards and Controls (NIBSC, South Mimms, Hertfordshire) and maintained in complete medium with the addition of 10-15 international units (IU/ml) of recombinant human interleukin -2 (rhIL-2) (a kind gift from Glaxo Ltd). The CTLLs required rhIL-2 for their survival and had to be fed every 3 days. The CTLLs were maintained by subculturing 1:10 (2 x 10^4 cells /ml) in complete medium with the addition of 10 -15 IU/ml of rhIL-2. The cells were cultured in tissue culture flasks standing upright in the incubator. The excess cells were either used in the IL-2 bioassay or were frozen down for storage in liquid nitrogen.

c) Hybridomas

Hybridomas secreting a variety of monoclonal antibodies (mAbs) were obtained (see Table 2.2 for a list of mAbs and their sources). The hybridomas were subcultured every 3-4 days depending on their individual growth rates. The non-adherent hybridoma cells and their culture medium (complete medium) were removed from the tissue culture flasks into a 50 ml tube. The cell suspension was centrifuged at 420g for 10 minutes and the supernatant was removed and stored as this contained the secreted mAbs. The cells were then subdivided with 10% of the cells being cultured further by resuspension 1:10 in complete medium. The excess cells were frozen down for storage in liquid nitrogen .

Preparation of cells for cryogenic storage.

To ensure an adequate supply of the many different cells types used in this research it was important to establish a good store of healthy cells which could be removed from long term storage in liquid nitrogen and cultured as required. When cells had been growing well in culture and were due for subculturing the excess cells if not required for experiments were removed and prepared for storage in liquid nitrogen. The method used was the same for all cells except the CTLLs (which is detailed separately).

After passaging the cells were washed in medium, centrifuged at 350g for 10 minutes and the medium removed. The cells were resuspended in 2ml 40% FCS in RPMI 1640. The cells were then put on ice and an equal volume of 20% DMSO in complete media added slowly. The cells were then subdivided between cryogenic storage vials at a density of 1 x 10^6 cells/vial. The vials were then cooled in a freezing box at -80°C overnight before being placed in long term liquid nitrogen storage.

CTLLs were prepared in a similar fashion except that the excess cells were stored in freshly made freezing mixture (20% FCS, 10% DMSO 70% RPMI 1640 and 1% rhIL-2) and were cooled more slowly. The vials of cells were cooled in a freezing box at -20°C for 8 hours then overnight at - 80°C before being transferred to liquid nitrogen storage.

To recover cells from cryogenic storage the cells were brought to 37°C quickly. The vial was swabbed with 70% ethanol to prevent contamination and the contents put into a tube containing 10ml of pre-warmed media (37°C). the cells were centrifuged at 350g for 5 minutes and the supernatant discarded. The cells were then resuspended in their appropriate medium (prewarmed to 37°C) and placed in culture at 37°C and 5% CO_2.

Monoclonal antibodies.

(Schlossman et al,. 1995)

The experiments in this research required the use of a number of different monoclonal antibodies (mAbs). Some of these antibodies were obtained by culturing the relevant hybridoma and collecting the secreted mAb found in the culture media. The presence of the secreted mAb was tested by using the supernatant as the primary mAb in the preparation of cells for FACS analysis and testing its ability to bind to its antigen on cells which expressed the antigen and also those which did not. If the mAb was present and the concentration of the mAb used in experiments had to be precisely defined the mAb was purified and quantified. Other mAbs were kind gifts from a number of different researchers. Details of all the mAbs used are shown in Table 2.2.

Table 2.2: Monoclonal antibodies (mAbs), their ligands, source and form of supply.

mAb	Ligand	source	form
OKT3	CD3 ε chain	A.T.C.C.	Hybridoma
OKT11	CD2	A.T.C.C.	Hybridoma
UCHM1	CD14	kind gift from P. Beverley	Hybridoma
L243	HLA-DR	A.T.C.C.	Hybridoma
BB1	CD80 (B7)	Kind gift from P.Linsley, Seattle	Ascites
IT2.2	CD86 (B70)	Kind gift from M. Azuma	Ascites
9.3	CD28	Kind gift from P. Linsley	Ascites

TS2/9	CD58 (LFA-3)	A.T.C.C.	Hybridoma
BU12	CD19	Kind gift from R. MacLennan	Ascites
8784	CD25 α chain	A.T.C.C.	Hybridoma

(A.T.C.C. - American Type Culture Collection, Maryland, USA)

Monoclonal antibody purification.
(Harlow and Lane, 1988).

The hybridoma culture supernatant containing the secreted mAbs was collected and the mAbs were precipitated by a two stage method using saturated ammonium sulphate (4.1M at 25°C) . The volume of culture supernatant obtained was determined and 0.5 of this volume of saturated ammonium sulphate (SAS) was added slowly with stirring and left at 4°C overnight. The mixture was centrifuged at 3000g for 30 minutes and the large proteins which had precipitated were discarded. A further 0.5 of the original supernatant volume of SAS was added and left overnight at 4°C. The mixture was centrifuged as before but the precipitate containing the mAbs was retained and redissolved in 0.1 volumes (of the original starting volume) of PBS. This solution was transferred to dialysis tubing and dialysed against 3 changes of PBS at 4°C. The mAbs were further purified using a protein G column where the unbound proteins present were washed through the column using 0.02M sodium phosphate pH 7.0. The bound mAb was eluted from the column using 0.1M glycine pH 3.7 into eppendorf tubes containing a small volume (20µl/ml eluant) of neutralising buffer (1.0M Tris-HCL pH 9.0). The concentration of protein in the aliquots of mAb obtained was determined spectrophotometrically by measuring optical density at 280nm. The monoclonal antibodies were tested using flow cytometry.

Purification of T cells from peripheral blood

(Boyum, 1964).

All blood samples were taken from healthy volunteers aged between 19 and 45. Blood (50ml) was collected into 2 tubes each containing 60µl of 5000 units/ml Heparin (Monoparin obtained from CP Pharmaceuticals Ltd). The blood was diluted 1:1 with PBS and separated on a Ficoll (1.077) density gradient (Lymphoprep from Nycomed (UK) Ltd) by centrifugation at 420g for 30 minutes.

The layer of mononuclear cells (PBMCs) (1 - 2 x 10^6 cells/ml blood) recovered from the gradient was resuspended in 50ml of complete media and centrifuged at 420g for 10 minutes. The supernatant was removed and the cells wash twice more by resuspending in 20ml of complete media and centrifuging at 420g for 5 minutes. The washed cells were then resuspended in 10ml of complete media.

The adherent sub-population of the mononuclear cells was removed by culturing the PBMCs in a small volume (usually 5ml) of medium in tissue culture plastic petri-dishes for 1 hour at 37°C. Non-adherent cells which include T cells were placed into a fresh tube and these cells were labelled using monoclonal antibodies to monocytes (UCHM1, anti-CD14, 500µl of 10µg/ml hybridoma supernatant), B cells (BU12 anti-CD19, 1:10 dilution of ascites) and activated T cells, monocytes and macrophages (L243 anti-HLA-DR, 500µl of 10µg/ml hybridoma supernatant) for 1 hour at 4°C on a rotator. Having been washed with complete media, the cells were incubated with 50µl sheep anti-mouse IgG coated magnetic beads diluted 1:10 in complete media for 1 hour at 4°C and the labelled cells were attracted to a magnet (Dynal) applied to the outside of the tube and the non-attracted T cells removed. The resulting T cell population was greater than 95% CD2 positive as shown by flow cytometry

Analysis of cells by Flow Cytometry (FACS)

(Ormerod 1990)

FACS analysis was used for two distinct purposes. One was to examine the surface molecules expressed on a number of different cell types e.g. the human surface markers whose cDNA had been transfected into CHO cells as well as surface markers on T cells such as CD3 , CD2 and CD28. FACS analysis was also used to test whether the hybridomas cultured were secreting their mAbs into the culture supernatant. This was achieved by using this supernatant as the source of primary mAb and testing a number of cells known to express the antigen for the mAb as well as non- expressing cells as a negative control.

Preparation of cells for FACS analysis

The cells (1-2 x 10^5 / tube) were washed in medium and incubated at 4°C for 20 minutes with 20 µl of heat inactivated human AB serum to block any surface Fc receptors. 50µl of the appropriate mAb at a concentration of

10μg/ml (or media as a negative control) was added and the cells incubated at 4°C for 60 minutes. The excess mAb was removed by washing the cells in complete media and the second layer antibody added - 50μl of sheep anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) at a concentration of 50μg/ml for all mAbs except BB1 which is an IgM antibody when sheep anti-mouse IgM conjugated with FITC was used. The cells were again incubated at 4°C for 60 minutes. The excess second layer antibody was removed by washing the cells in complete medium and the cells were resuspended in 200μl PBS for immediate analysis. FACS analysis done at a later date was performed on cells resuspended in 100ul 2% FCS/PBS, fixed with 100μl of 2% (w/v) paraformaldehyde in PBS and stored at 4°C. The labelled cells were diluted with FACS sheath fluid (Becton Dickinson) and analysed using a Facstar Plus (Becton Dickinson) with a 100mW 488nm argon laser. Information about their forward scatter (FSC), side scatter (SSC) and the fluorescence intensity of the labelling (FLI) was obtained using the LYSIS II program.

T cell proliferation assays

(Waldmann et al., 1987).

Preparation of costimulatory cells

Transfectants were trypsinised and washed twice with PBS to remove traces of FCS. Cells were fixed for 2 minutes with 0.025% (v/v) gluteraldehyde (Sigma) in PBS. The fixation was stopped by the addition of 200mM glycine and the cells washed twice in FCS containing medium to remove excess gluteraldehyde. The cells were then counted and resuspended in complete medium at the concentration required for each experiment. On all occasions, the transfectants used were cultured alone in the proliferation experiments with tritiated thymidine to measure background proliferation.

Proliferation of Purified T cells

T cells purified from PBMCs were washed in media, counted and plated out in 96- well flat bottomed plates (normally at $5x10^4$ cells/50μl/well). Where the T cells were stimulated using the mAb OKT3, the 96-well plate was pre-incubated with mAb at the appropriate concentration (usually 1μg/ml) in PBS overnight at

room temperature before the unbound mAb was washed off. The fixed transfectants used to provide costimulation were added at the appropriate concentration (normally 1-2x 10^4 cells/50µl/well). The final volume in each well of the assay was routinely 200µl. Each culture was plated out in triplicate. The assay was incubated at 37^0C and 5% CO_2. At the time point required by the particular assay, 50µl of culture supernatant from each well was removed and stored for IL-2 determination. The wells were pulsed with 1µCi ^{3}H thymidine (specific activity 6.7 Ci/mmol) (Amersham International plc) (50µl volume) and incubated for 18 hours. The pulsed wells would either be removed to another plate and frozen for subsequent measurement of ^{3}H thymidine incorporation or if the whole plate was being assayed at one time point, the plate would be harvested and thymidine incorporation counted. The cells were lysed, the radiolabelled DNA trapped onto glass-fibre filter mats using a semi-automatic cell harvester (Skatron Ltd). Each disc of filter mat corresponding to each well in the 96-well plate was put in a scintillation vial and 0.5ml of scintillant added (Optiscint HiSafe obtained from Wallac). The amount of radioactivity incorporated in each well was measured using a scintillation counter (LKB beta counter from Wallac Ltd).

IL-2 Bioassay

(Gillis et al., 1978).

The CTLLs were used after 3 days in culture when their supply of added human rhIL-2 was depleted. The cells were washed twice in culture media and a viability count made using the Trypan Blue exclusion method. Providing viability was greater than 80% the cells were used. 50µl of the culture supernatant from each sample (diluted 1 in 2 and 1 in 10 with complete medium) was plated out in triplicate in a 96-well round bottomed plate and 50µl of a CTLL suspension in media at 1 x 10^5 cells/ml added to each well. A standard curve was always used in the assay using rhIL-2 (NIBSC) at 0.019 -10 IU/ml. The cells were left in culture overnight at 37^oC and 5% CO_2 before 0.5µCi of ^{3}H thymidine (specific activity 6.7 Ci/mmol) was added to each well. The cells were incubated at 37^oC for a further 4 hours. The level of ^{3}H thymidine incorporation was determined by harvesting the DNA from the cells onto filter mats and counting using a liquid scintillation beta counter. The amount of IL-2 present in the supernatant from the proliferation assay was calculated using the calibration curve. An example of the standard curve produced is shown in Figure 2.1 below.

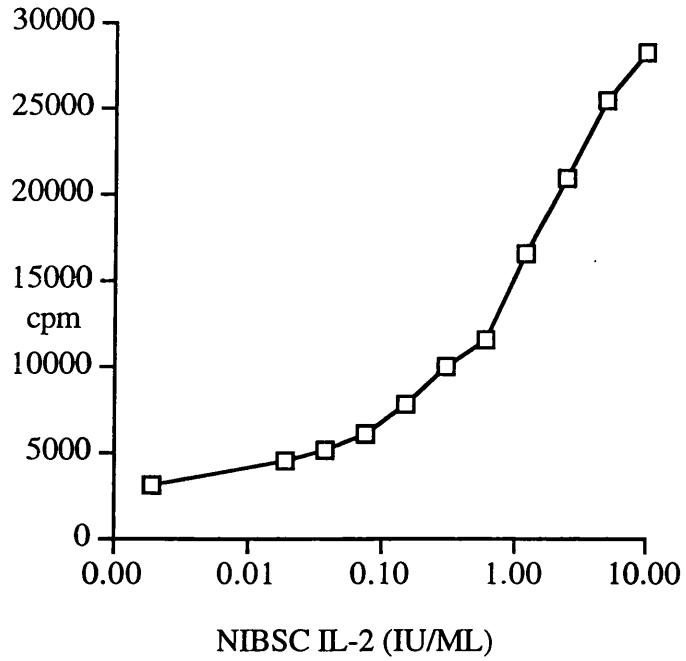


Figure 2.1 : A standard curve for CTLL2 proliferation.
Using known concentrations of human rhIL-2 to provide a calibration curve the concentration of IL-2 present in the supernatant of T cell proliferation assays could be determined if the values obtained were on the linear portion of the standard curve.

T cell blasts

Production of T cell blasts

The addition of specific mitogens to PBMCs in culture stimulates the T cells present to proliferate. Within a few days most of the other mononuclear cells have died and the culture consists predominantly of T cell blasts. The mitogens used to stimulate the PBMCs were either

a) SEB at a final concentration of 1µg/ml

or

b) PHA at a final concentration of 10µg/ml

or

c) PMA at a final concentration of 50ng/ml and Ionomycin at a final concentration of 1µM.

Following stimulation the cells were cultured in complete medium without the addition of exogenous IL-2.

Culturing of T cell blasts

T cell blasts were cultured in complete medium and were given fresh medium as required (normally every 2-3 days) with the maximum duration in culture being 10 days post-mitogenic stimulation.

Proliferation of T cell blasts

(Waldmann et al., 1987).

T cell blasts were washed in medium to remove any cytokines present in the culture media, counted and plated out in 96-well round bottom plates (except when stimulated with OKT3 when mAb-coated flat bottom plates were used) at the appropriate concentration (normally $5x10^4$ cells/50µl/well). The fixed transfectants used to provide stimulation were prepared and added as for pure T cell proliferation assays. Each culture was plated out in triplicte.The total volume in each well of the assay was made up to 200µl with medium. The plates were incubated at 37°C and 5% CO_2 for two days before 50µl of supernatant was removed for IL-2 determination and 1µCi of ^3H thymidine (specific activity 6.7 Ci/mmol) added. After overnight incubation with the radiolabelled nucleotide the cells were harvested and ^3H thymidine incorporation was measured as for purified T cell proliferation assays.

Inhibitors

Handling and storage

The fungal metabolite wortmannin was used to inhibit intracellular signalling in T cell proliferation assays. As this chemical is thought to be carcinogenic, mutagenic and possibly teratogenic it was handled wearing appropriate safety clothing and in a laminar flow cabinet. Wortmannin was dissolved in ethyl acetate at a concentration of 20mM, aliquoted into Eppendorf tubes and stored at -20°C.

Using wortmannin in proliferation assays

As wortmannin is unstable in aqueous solutions, when being used in proliferation assays, it was diluted to the required concentrations in cultured media immediately prior to use.

Cytokine mRNA determination.

The recipes for the various solutions used for cytokine mRNA determination are given in Appendix 3.

RNA extraction.

RNA was extracted from cells using the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). As RNA is easily degraded due to the presence of the enzyme RNase on hands and in solutions all manipulations were carried out wearing gloves and all reagents used were dedicated to RNA work only.

Cells (approximately 10^6 cells/100µl RNA extraction solution) were lysed in RNA extraction solution (4M guanidinium thiocyanate, 25mM sodium citrate pH7, 0.5% sarcosyl, made up in diethylpyrocarbonate (DEPC) - treated distilled water with 0.1 M 2 - mercaptoethanol being added immediately prior to use) in a microfuge tube to a final volume of 500µl. The following reagents were added sequentially with mixing by inversion between each addition - 50µl 2M sodium acetate pH4, 500µl water saturated phenol (Appligene) and 100µl chloroform - isoamyl alcohol (49:1). The mixture was vortexed for 10 seconds and incubated on ice for 15 minutes prior to centrifugation at 12,000g for 10 minutes at room temperature. The top aqueous layer was removed to a fresh microfuge tube and an equal volume of chloroform - isoamyl alcohol added. The mixture was then vortexed and centrifuged at 12,000g for 5 minutes at room temperature. This chloroform - isoamyl alcohol extraction was repeated and the final aqueous layer precipitated overnight at -20°C by the addition of an equal volume of isopropanol. The precipitate was centrifuged at 12,000g for 10 minutes at room temperature and the RNA redissolved in 150µl of RNA extraction solution. The RNA was reprecipitated at -20°C overnight by the addition of 150µl of isopropanol. The RNA was centrifuged at 12,000g for 10 minutes at room temperature and the pellet

washed twice with 70% ethanol and once with 100% ethanol before drying under vacuum for approximately 15 minutes. The RNA was redissolved in 50µl DEPC - treated water.

RNA quantitation

The concentration of RNA was determined spectrophotometrically. The RNA was diluted 1:24 in water and the optical density measured at 260nm and 280nm. The concentration of RNA was calculated using the following formula:-

A_{260} x 40 x dilution = µg/ml RNA.

The purity of the sample was determined from the ratio A_{260}/A_{280}, with pure samples having a ratio of 2.0. Lower ratios indicated proteins present or contamination by phenol.

Agarose gel electrophoresis

Horizontal submarine gel electrophoresis was used to analyse the RNA extracted to determine its integrity and also to identify DNA fragments produced by Reverse Transcription - Polymerase Chain Reaction (RT-PCR). A 1%(w/v) gel (for RNA analysis) or 2%(w/v) gel (for RT-PCR analysis) was produced by dissolving agarose in Tris - acetate ethylenediaminetetraacetic acid (EDTA) buffer (TAE) (0.04M Tris base, 5mM sodium acetate and 1mM disodium EDTA). The agarose / buffer mixture was heated in a microwave to allow the agarose to dissolve and then cooled to approximately 60°C before the addition of ethidium bromide (final concentration 0.5µg/ml). The cooled agarose gel was poured into a gel mould and allowed to set. The gel was covered with TAE buffer and the samples to be analysed were loaded onto the gel in the appropriate loading buffer (20% w/v Ficoll (Type 400) and 0.2% w/v bromophenol blue for RNA and 25% w/v Ficoll (Type 400), 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol for DNA). The samples were electrophoresed at 100mA until the leading edge of the loading dye was approximately half/three quarters of the way down the gel. The RNA or DNA was visualised using a UV transilluminator.

When the DNA fragments from the RT-PCR were being analysed a ϕX 174 DNA digested with Hae lll (Northumbria Biologics Ltd or Promega Ltd) was run at the same time to allow sizing of the fragments. The size of the markers in base pairs (bp) are as follows -1,358bp, 1078bp, 872bp, 603bp, 310bp, 281bp, 271bp, 234bp, 194bp, 118bp and 74bp.

Reverse transcription of RNA

RNA was reversed transcribed to produce cDNA prior to amplification by PCR. The reaction was carried out in silanised microtubes to prevent the cDNA from sticking to the tube. Approximately 500ng of total RNA was mixed with 10mM dithiothreitol, 0.5mM each of dATP, dCTP, dGTP and dTTP, 1.5μM pd(T) 12 - 18, 30 units RNAguard (RNase inhibitor , all from Pharmacia Biotech Ltd), 400 units M-MLV reverse transcriptase (Life Technologies Ltd) and 1 x RT buffer (supplied with the enzyme). The total volume was made up to 30μl with DEPC - treated water. The mixture was incubated at 37°C for 60 minutes then heated at 95°C for 10 minutes. The cDNA was then put on ice if being used immediately for PCR or stored at -20°C.

Polymerase chain reaction (PCR)
(McPherson et al., 1992)

Although all the cDNA produced by the reverse transcription of the total cell RNA was used in the PCR process only the fragments of interest were amplified. This was achieved by using primer pairs specific to the DNA sequences under investigation. The primer pairs used were able to amplify cDNA produced from RNA for IL-2 and Glyceraldehyde - 3 - phosphate dehydrogenase (GAPDH) respectively. Their sequences are shown below -

IL-2	5'	GAATGGATTTAATAATTACAAGAATCCC 3'
	3'	TGTTTCAGATCCCCTTTTAGTTCGAC 5'
GAPDH	5'	GGTGAAGGTCGGAGTCAACGG 3'
	3'	GGTCATGAGTCCTTCCACGAT 5'

The products produced by the PCR amplification process had molecular weights of 229bp and 520bp for IL-2 and GAPDH respectively. The primer pairs span introns in the gene sequence so ensuring that any contaminating genomic DNA would not be amplified or would have a much higher molecular weight.

PCR reactions were carried out in a final volume of 100µl, made up as follows -

10µl of Taq buffer (supplied with enzyme)
200µM each of dATP, dGTP, dCTP and dTTP.
100nM each of 5' and 3' primers
1.25mM $MgCl_2$
2.5 units Taq polymerase (Northumbria Biologics Ltd or Promega Ltd)
2µl cDNA reaction mix

The reaction mixture was overlaid with 40µl of mineral oil to minimise evaporation and amplification carried out for 30 cycles on an Astec PC-700 machine using the following programme -

1 minute at 94°C
1.5 minutes at 55°C
1.5 minutes at 72°C

This was followed by 10 minutes at 72°C and the samples were stored at 4°C until examined using gel electrophoresis.

Data Analysis

All experiments were carried out a minimum of three times using different blood donors on each occasion. The data presented in the results section are representative of each set of experiments and the number of experiments carried out is shown in the figure legend.

The data from proliferation assays represent the mean values from triplicate cultures and the error bars represent standard errors. Where error bars are not visible this is due to the scale of the graph being too large relative to the magnitude of the error.

Chapter 3

CD28 costimulation in purified T cells

Generation of CD86 transfectants.

In order to investigate the role of CD28 in T cell activation and to be able to use the natural ligands for CD28 to provide costimulatory signals to T cells, transfected cells expressing the CD28 ligands CD80 and CD86 were generated in the laboratory. However the newly transfected cells have to be grown and selected to produce a stable expressing cell line which can be used repeatedly in different experiments so allowing comparisons between experiments to be made. CHO cells were chosen as the vehicle for transfection as they have been used successfully by many groups to express human surface molecules and are unreactive to human T cells.

Although the method used to transfect the CHO cells (electroporation) is considered to be a highly effective technique not all the CHO cells were transfected with the cDNA or expressed the required protein. Surface expression of the human CD86 molecule on the CHO cells was determined by FACS analysis. The level of expression of CD86 on newly transfected CHO cells (24 hours after transfection) is shown in Figure 3.1b (the background fluorescence level is shown in Figure 3.1a). This shows that 39% of the cells expressed CD86 (B70) on their surface which was quite a high level of transfection - sometimes less than 10% of transfected cells are shown to express the required protein. The FACS analysis also shows a highly variable level of expression of CD86 on the transfected CHO cells with the majority having low levels of expression. The fluorescence intensity (FL1) shown on the x - axis is a measure of the number of CD86 molecules expressed on the surface of each CHO cell - the greater the number of CD86 molecules on the cell surface the higher the fluorescence intensity detected (i.e. FL1 value). The wide range of FL1 values shown in figure 3.1b indicates that the cells transfected expressed CD86 at different levels and the general level of the expression was low.

Cells transfected with foreign cDNA are highly unstable with the vast majority of the cells either not expressing the required protein or the foreign cDNA being destroyed or expelled during cell division. In order to produce a high expressing stable transfectant, the transiently expressing CHO cells had to be sorted and the required cells selected. This was achieved in three ways. Initially, the cells expressing CD86 were selected using a magnetic bead system to separate those cells expressing CD86 from the non-expressors. This is a quick and easy method to separate out large numbers of cells, which

express the required surface marker, for further selection. The cells expressing CD86 were cultured and their surface expression analysed by FACS. The cells were then reselected using the magnetic bead method twice more and the resulting improvement in the percentage of cells expressing of CD86 can be seen in Figure 3.1c - nearly 68% compared to the initial 39% before immunomagnetic selection. However this method was of limited use as all cells expressing CD86 were selected using a magnetic bead system regardless of their level of expression. To be able to distinguish and separate out transfectants expressing high levels of CD86 (as shown by FACS analysis) a second method was employed - FACS sorting.

This is a more complex method of sorting cells where specific parameters such as the level of expression of the CD86 were used to separate out the required cells from the rest of the transfectants . As a result only small numbers of cells could be processed using this method (compared to the more crude magnetic bead selection method).The sorted transfectants were cultured and examined for their surface expression of CD86. This basic method of FACS sorting had resulted in an increase in the number of cells expressing CD86 (95.6% compared to 68.48% following magnetic bead selection - Figure 3.1d) and the selected cells showed a high level of expression of CD86 with a mean fluorescence to 146.5 (as compared to a mean fluorescence of 58.61 in transfectants sorted using magnetic beads).

However there was still variation in the level of expression of CD86 on individual cells as the cell population was still polyclonal. To overcome this variation in surface expression of the transfectants cultured, a third method of selection was used which involved not only sorting the cells expressing CD86 but also directly cloning them using an Automatic Cell Deposition Unit attached to the FACS machine. The cells were again selected using criteria such as the level of expression of CD86 on their surface but the individual cells selected were collected separately and cultured as individual clones. This was carried out in as sterile conditions as possible. The cloned transfectants were initially cultured in 96 well tissue culture plates, the cultures expanded and the cells screened for surface expression on the FACS. Not all the clones successfully grew or expressed CD86 but a number of high expressing clones were cultured for use in the T cell activation experiments.

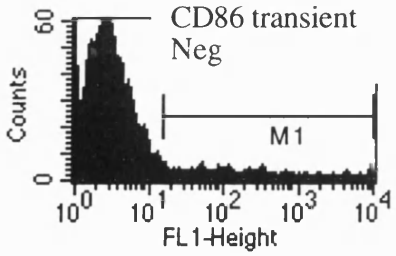
FACS analysis of the surface expression of two of CD86 clones is shown in Figures 3.1e and 3.1f. This demonstrates that although both clones were

expressing CD86 the clone G8 had a much more uniform level of expression when compared to that of clone F9. It is likely that F9 was not a single clone but possibly biclonal - i.e. two cells were sorted into one of the wells on the 96 -well plate and both were cultured and expanded together. This would explain the variation in the level of surface expression of CD86. Transfectants from clones such as Clone G8 were selected for use in future experiments. All the transfectants used (CD80, CD86 and CD58) have been screened and cloned to ensure similar levels of expression of their particular human surface molecule.

The transfectants used in any experiment were routinely screened to determine not only the level of expression of their own surface ligand but to ensure that they did not express any other surface molecules which could have an effect on the results of the experiment. An example of this is shown in Figure 3.2. The CD80 transfectant has been screened using mAbs to CD80 (BB1), CD86 (IT2.2), CD58 (TS2/9), and HLA-DR (L243) and only CD80 shows a fluorescence intensity (FL1) greater than the background level. This clearly demonstrates that the transfectant expressed only the desired surface molecule.

Having established transfected cell lines which could be used to provide costimulatory signals to T cells, it was necessary to establish a method of providing the initial stimulus via the T cell receptor.

a)



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Marker	% Total	Geo Mean
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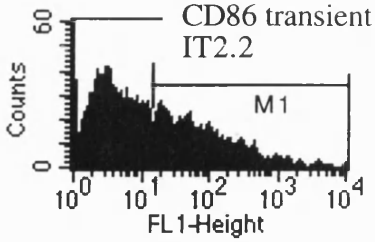
M1	7.57	125.30

b)



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Marker	% Total	Geo Mean
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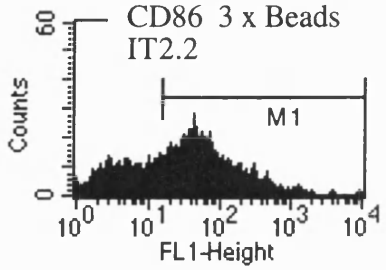
M1	39.38	64.47

c)



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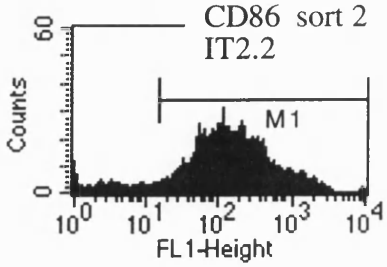
Marker	% Total	Geo Mean
All	100.00	27.08
M1	68.48	58.61

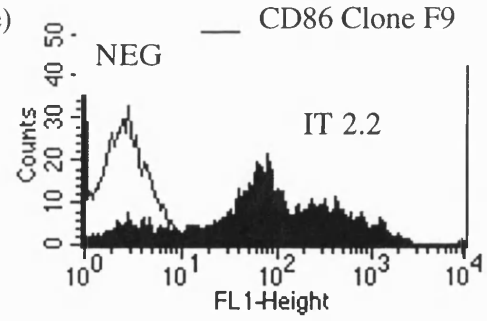
d)



Total Events: 5000

Marker	% Total	Geo Mean
All	100.00	124.71
M1	95.62	146.54

e)



f)

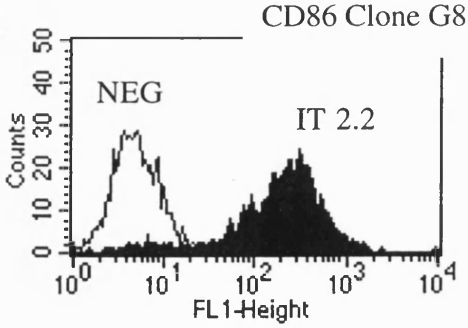


Figure 3.1 The effect of cell selection processes on the surface expression of CD86 on CHO cells transfected with CD86 cDNA.
Transfectants were stained with IT2.2 (anti-CD86 mAb) and analysed by FACS. The surface expression of CD86 on transfectants 24 hours after insertion of CD86 cDNA is shown in figure b), the effect on surface expression following selection using magnetic beads is shown in c) and cell sorting selection is shown in d). The background fluorescence level is shown in a). Figures e) and f) show the differences in surface expression that can occur when the transfectants were cloned.

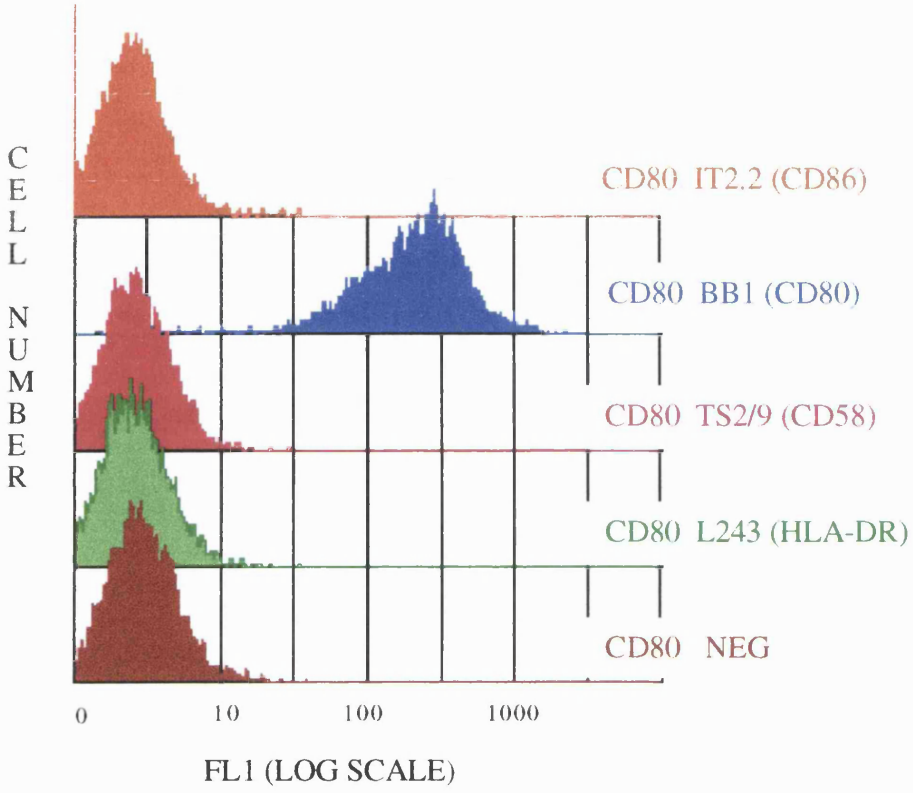


Figure 3.2 FACS analysis of surface expression of CD80 on transfected CHO cells.

CD80 transfectants were stained with mAbs to HLA-DR (L243), CD58 (TS2/9), CD86 (IT2.2) and CD80 (BB1). The cells were then examined by FACS to determine which of these surface molecules was being expressed on the transfectants. The background level of fluorescence is also shown. The data are representative of a single experiment (n>10).

T cell stimulation by anti-CD3 mAb.

The normal primary stimulus in T cell activation is the interaction of the TCR-CD3 complex of the T cell with the MHC - antigen complex on the antigen presenting cell (APC). However this interaction only occurs in those T cells which express the appropriate T cell receptor and activation of such a small number of T cells would be difficult to detect. A more general signal was required to stimulate the heterogeneous TCR specificity found in peripheral T cells and this was provided by a mAb (OKT3) to the ε chain of the CD3 complex which associates with the TCR. To ensure that this signal was consistent between experiments the mAb produced by the hybridoma cells was purified using Protein G affinity chromatography and a known amount used to provide the primary stimulus .

The primary signal was supplied by coating the mAb diluted in PBS onto 96-well flat bottomed plates. The efficiency of coating the plates was detected using an anti-mouse IgG alkaline phosphatase conjugate in an enzyme-linked immunosorbant assay (ELISA) (see Appendix 4 for the method). The results are shown in Figure 3.3. The results demonstrated that the optimal time of those examined for coating plates with OKT3 was 24 hours at room temperature and this was used in all subsequent experiments.

The final component of the T cell activation experiments was the purified T cells. It was important to ensure that the method used to generate these T cells was efficient and reliable. In this way the purity of the T cells obtained would be consistent so that variation in purity of T cells between experiments would be controlled.

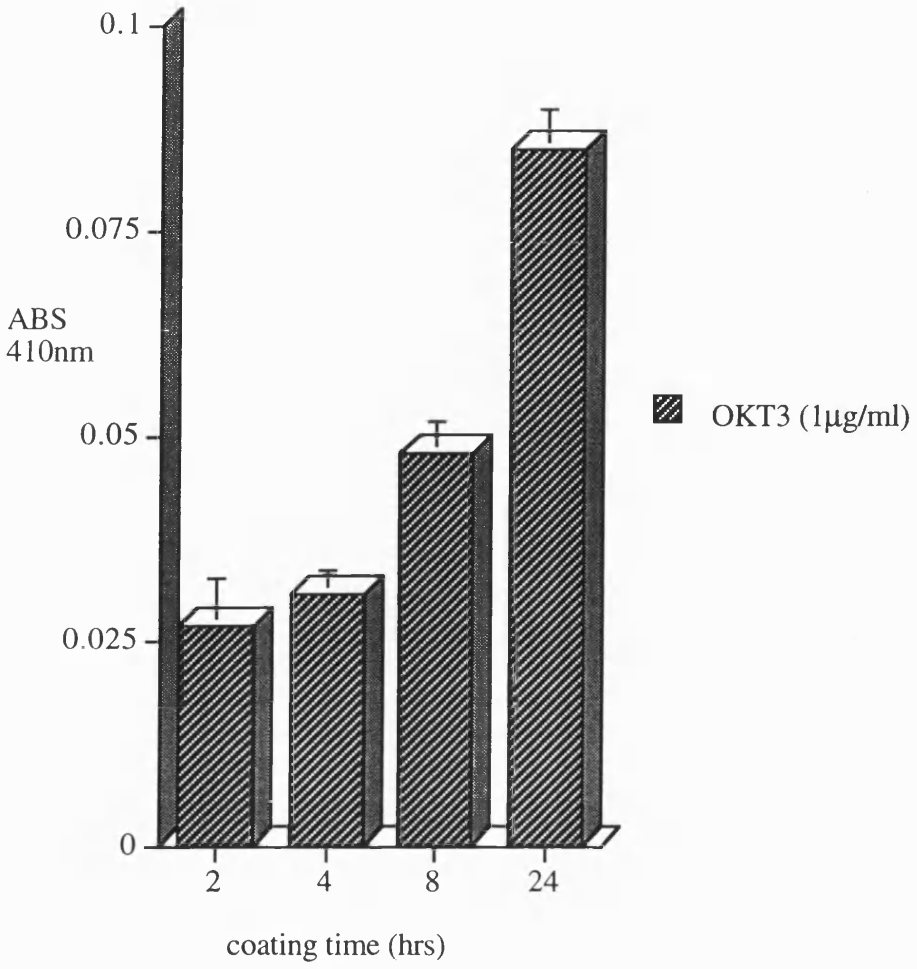


Figure 3.3 : Time dependency coating of 96 -well plates with the mAb OKT3. A 96-well flat bottomed plate was coated with the mAb OKT3 at 1 µg/ml (diluted in PBS) for various times at room temperature. The amount of mAb which adhered to the plate was detected using an anti-mouse IgG alkaline phosphatase conjugate and disclosed with p-nitrophenyl phosphate (pNPP) which produces a coloured product that can be measured spectrophotometrically at 410nm. The data are from triplicate samples from a single representative experiment (n = 3).

Analysis of purified T cells

The purity of the T cells used in the costimulation assays was determined by FACS analysis using mAbs to detect the presence of contaminating monocytes (UCHM1 for CD14), B cells (BU12 for CD19) and activated T cells, monocytes and macrophages (L243 for HLA-DR which is upregulated on activation). A typical FACS result is shown in Figure 3.4a. The level of expression of these surface molecules was compared to a negative control (when no primary mAb is used to give the background level of fluorescence). The level of expression of CD19 , CD14 and HLA-DR was similar to the background level of fluorescence demonstrating the lack of contaminating cells in the T cell sample.

The purified T cells were then analysed by FACS to examine the expression of a number of surface molecules as shown in Figure 3.4b. This was to ensure that the cells obtained were T cells and that the sample did not contain T cells which were already activated. The levels of expression of CD3, CD2, CD28 and CD25 on the surface of the T cells (using the mAbs OKT3, OKT11, 9.3 and 8784 respectively) were determined. The purified T cells were found to express CD3, CD2 and CD28 but not CD25 which is a marker of activation (Figure 3.4b). As not all T cells express CD3, the percentage of cells expressing CD2 was taken as an indication of the purity of the T cells obtained. On all occasions this was at least 95% (Figure 3.5b). The background fluorescence level is shown in Figure 3.5a. FACS analysis of purified T cells was carried out before each experiment to ensure that the purification process had been successful and that any variation in the experimental results was not due to the presence of contaminating cells.

Having established appropriate reagents initial experiments were undertaken to determine whether CD80 transfectants would stimulate T cells via their CD28 receptor in conjunction with a signal via the TCR- CD3 complex and activate the T cells.

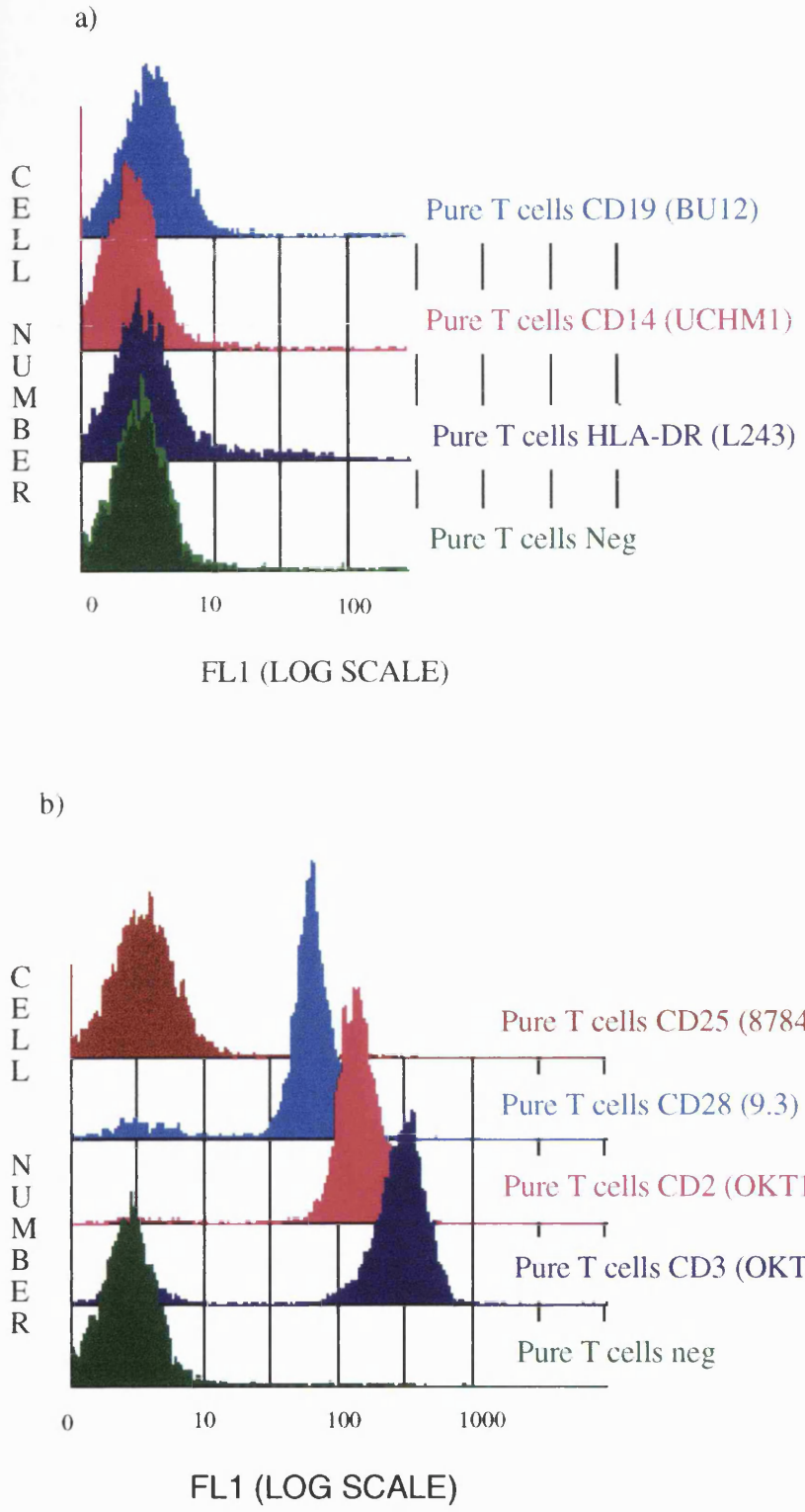
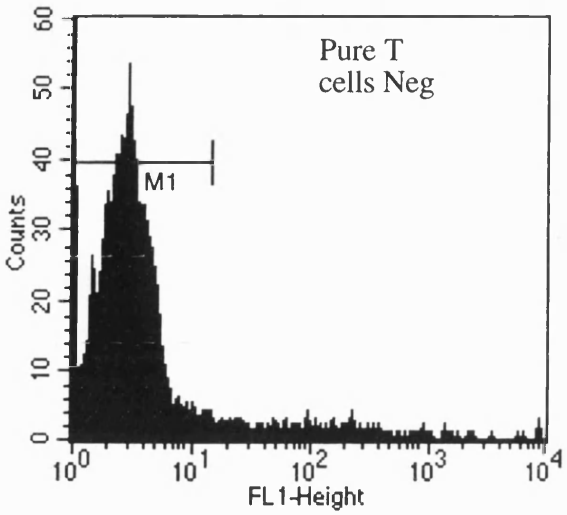


Figure 3.4. FACS analysis of Purified T cells.
a) Purified T cells were analysed by FACS to determine whether there was contamination by monocytes , B Cells or activated T cells.
b) Purified T cells were analysed by FACS to determine their surface phenotype using mAbs to CD3, CD2, CD28 and CD25. The data are representative of a single experiment (n>10).

a)



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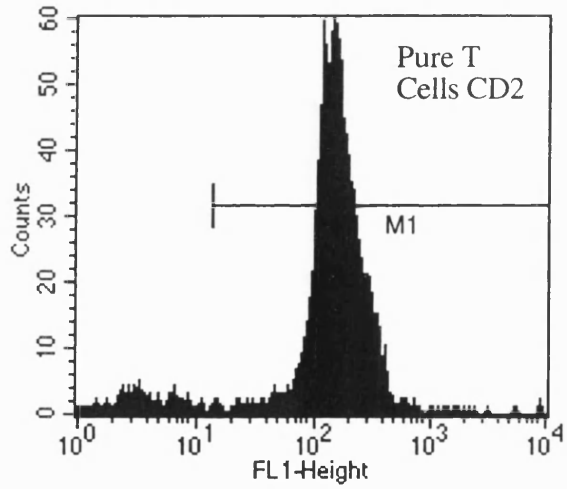
M1	94.10	2.78

b)



Total Events: 5000 X Paramete

Marker	% Total	Geo Mean
All	100.00	127.59
M1	95.46	151.04

Figure 3.5 Analysis of purity of T cells obtained from human blood. Purified T cells from human blood were stained with OKT11 (anti- CD2 mAb) and analysed by FACS. The percentage of positive cells was determined by setting a gate on the negative control corresponding to approximately 95% of all cells (a). Using the same gate parameters the percentage of positive cells outside this area was determined (b). The facs data shown are from a representative experiment (n>10).

CD28 costimulation of purified T cells

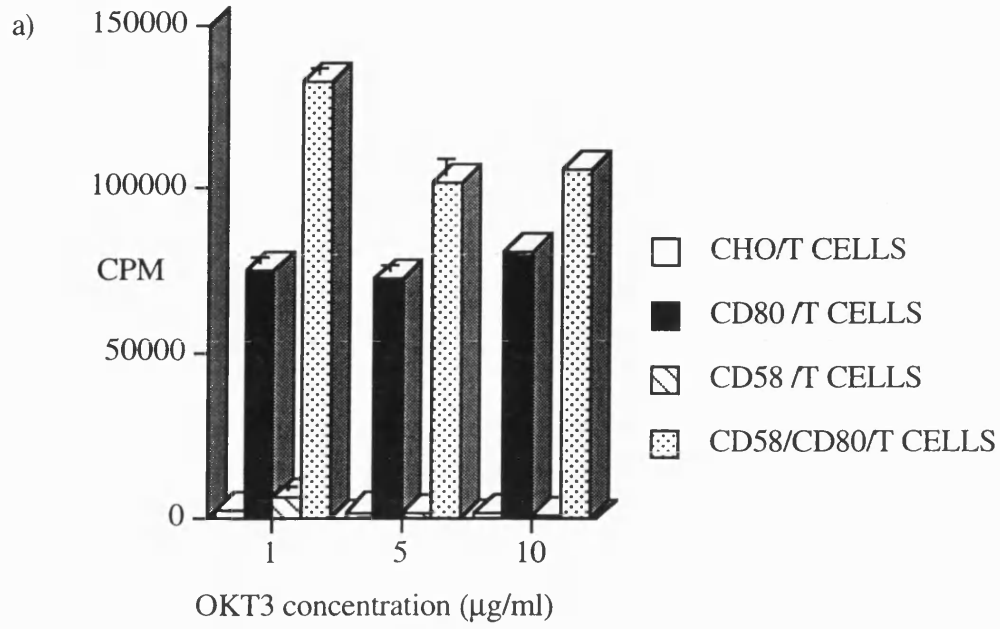
To determine whether CD28 ligation by CD80 transfectants could generate the necessary costimulatory signals for T cell activation, purified unactivated T cells were stimulated using a monoclonal antibody (mAb) to the ε chain of the CD3 complex (OKT3) and signals provided by Chinese Hamster Ovary (CHO) cells transfected with CD80 (B7), and/or CD58 (LFA-3) with the untransfected parental cell as a control. Three different concentrations of OKT3 were used and the resulting T cell activation was measured in terms of T cell proliferation (via 3H thymidine incorporation) and IL-2 production (using a CTLL bioassay).

The proliferation results are shown in Figure 3.6a. The T cells were stimulated to proliferate at all three concentrations of OKT3 only when CD80 was present on the costimulatory cell. The parental CHO cell line did not provide co-stimulatory signals and the OKT3 mAb was insufficient to activate the T cells. The CD58 transfectants were also unable to provide co-stimulation to the T cells and it is possible that this may have been due to lack of interaction between the CD58 transfectant and its ligand CD2 on the T cell. However when the double transfectant CD58/CD80 was used to provided costimulation a higher level of proliferation was generated than when the single transfectant was used. This suggests that CD58 expressed on the double transfectant was able to interact with its ligand CD2 on the T cells so increasing adhesion between the T cells and the transfectants resulting in a higher level of proliferation. This increased level of proliferation in T cells costimulated with the double transfectant may also have been due to increased signalling via the CD3 complex following CD2 ligation by CD58 as this is the known pathway of CD2 signalling (Howard et al., 1992). There was little difference in the level of proliferation obtained using increasing concentrations of OKT3 to coat the 96-well plates suggesting that the level of binding to the T cells was saturated at 1μg/ml. As a result 1μg/ml was used routinely in all experiments.

The proliferation results were paralleled by the IL-2 production figures (Figure 3.6b) with IL-2 only being produced when CD80 was present on the costimulatory cell either as a single or double transfectant. However although the double transfectants produced a higher level of proliferation than the single transfectant there was little difference in the level of IL-2 detected. It is likely that the IL-2 produced when costimulation was provided by the double transfectant was used more rapidly by the T cells resulting in higher levels of

proliferation. As the bioassay used to detect IL-2 production measures only unbound IL-2 any increase in IL-2 production when the double transfectant was used would not be detected if it was being taken up by the rapidly proliferating T cells.

To ensure that the proliferation observed using CD80 transfectants as costimulators was not due to any mitogenic effects of the transfected CHO cells, purified T cells were cultured with transfectants in the presence or absence of OKT3. The results in Figure 3.7 show that CD80 provides co-stimulation in the presence of OKT3 but does not induce T cell proliferation on its own. The background levels of proliferation for T cells and fixed transfectants were also determined. Proliferation of T cells and fixed transfectants was generally less than 1000cpm and IL-2 production could not be detected. This demonstrated both the necessity of appropriate stimuli to activate purified T cells and the effectiveness of the transfectant fixation method.



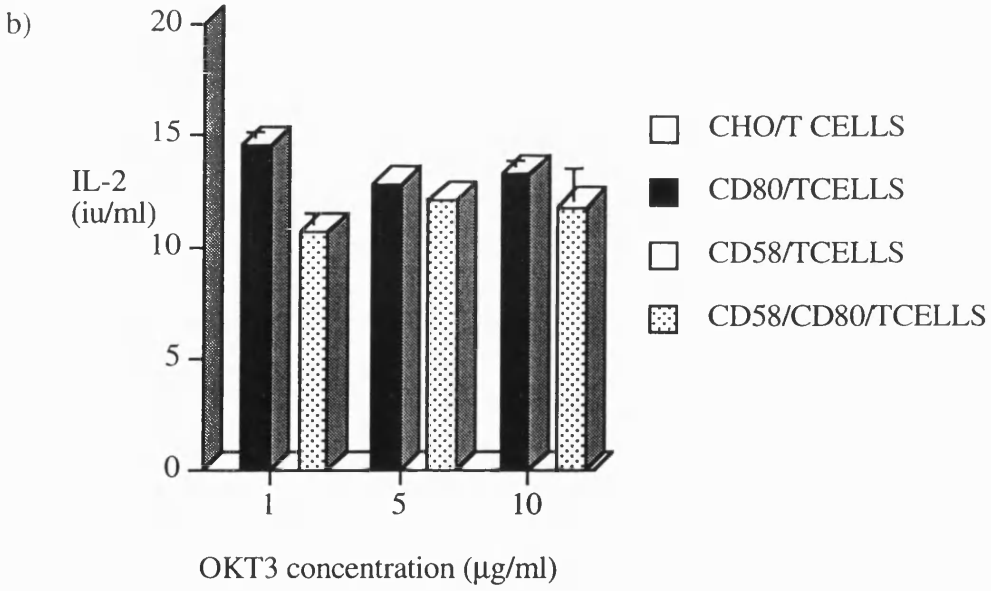


Figure 3.6 : OKT3 and CD80 stimulation of purified T cells.
Purified T cells (2 x 10^4) were stimulated at three concentrations of OKT3 in the presence of transfected CHO cells (2 x 10^4). This resulted in (a) T cell proliferation, measured after 48 hours using ^{3}H Thymidine incorporation and (b) IL-2 production measured after 48 hours using a CTLL bioassay.The data are from triplicate samples from a single representative experiment (n = 3).

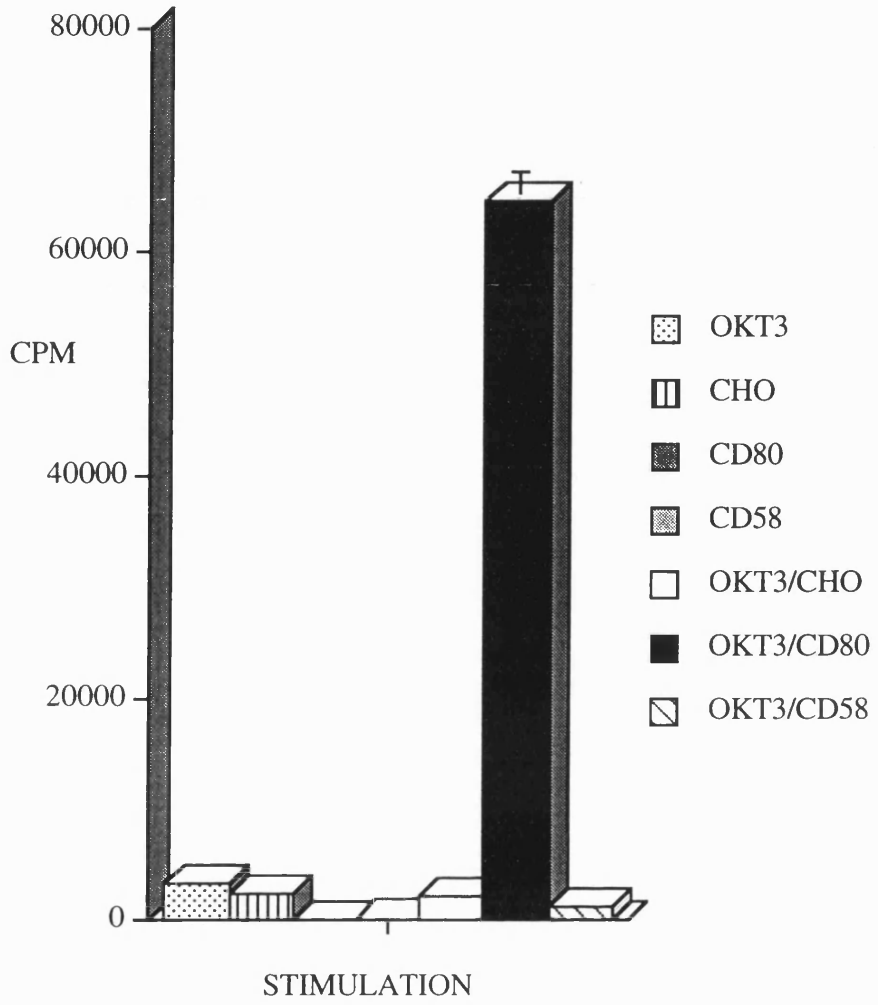


Figure 3.7 : Costimulation of purified T cells by OKT3 and CD80.
Purified T cells (2 x 10^4) were cultured with transfectants (2 x 10^4) in the presence or absence of OKT3 (1µg/ml) for 72 hours. Proliferation was measured by ^3H thymidine incorporation. The data are from triplicate samples from a single representative experiment (n = 5).

These initial experiments established that ligation of CD28 by CD80 expressed on transfected CHO cells in the presence of OKT3 was essential for T cell activation to occur as demonstrated by T cell proliferation and IL-2 production. T cell activation could not occur unless both these signals were present and ligation of CD2 by CD58 in the absence of CD28 - CD80 interactions could not provide the necessary costimulatory signals. Thus

these data were consistent with other reports that CD28 was necessary for T cell activation (e.g. Linsley et al., 1991a).

Kinetics of T cell activation following CD28 costimulation

Having established that CD28 could provide costimulatory signals to T cells (following ligation by CD80 transfectants) to induce T cell activation, it was then possible to examine CD28 costimulation in greater detail. The effect of CD28 costimulation on the kinetics of T cell activation was studied by examining the proliferative response and IL-2 production of purified T cells over a number of days following culturing with transfectants (CD80, CD58 or the CHO parental cells) in the presence of OKT3.

The results (Figure 3.8a) demonstrated that CD80 transfectants provided the necessary costimulatory signals to T cells with maximum proliferation occurring by 120 hours in culture. Neither the untransfected parental cell nor the CD58 transfectants were able to provide costimulatory signals to induce T cell proliferation.

When production of IL-2 by the T cells was examined, only the CD80 transfectants were able to provide the necessary costimulatory signals for IL-2 production. When CD58 transfectants or untransfected CHO cells were used to provide costimulation, they were unable to induce the T cells to produce IL-2. Under these conditions, the maximum level of IL-2 detected occurred much earlier than maximum proliferation - after 48 hours in culture (Figure 3.8 b).

The fact that CD80 ligation of CD28 resulted in IL-2 production was not unexpected as it had already been shown that CD28 signalling results in a two fold increase in the rate of IL-2 transcription as well as increasing the stability of IL-2 mRNA (Lindsten et al., 1989; Fraser et al., 1991). More recent research has demonstrated that CD28 signalling can induce a number of IL-2 gene transcription factors - CD28RC, NF-kB and AP -1 (Fraser et al., 1993; Edmead et al., 1996).
The data from figure 3.8 demonstrated that IL-2 production preceded T cell proliferation. This is not unexpected as T cells proliferate in response to IL-2. To be able to do this T cells must express the IL-2 receptor (which is not constitutively expressed on T cells) and signals generated by CD28 are known to upregulate IL-2R β chain (Cerdan et al., 1995). As a result of CD28 signalling, IL-2R bearing T cells were generated and were able to respond to

the IL-2 already produced, therefore increased proliferation was detected after IL-2 production had reached its maximum. The loss of IL-2 detected as proliferation was increasing was probably due to the cytokine being used by the IL-2R bearing T cells.

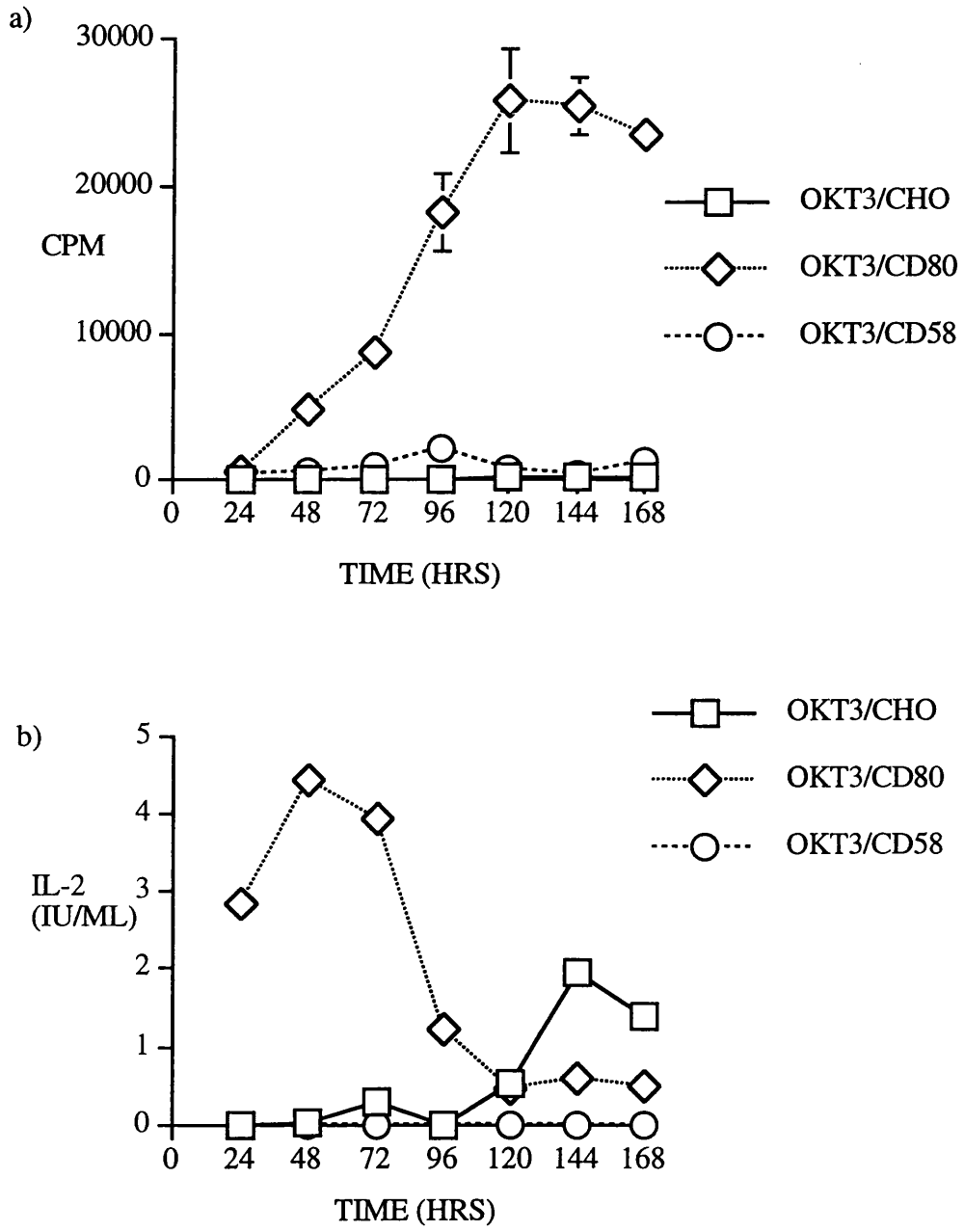


Figure 3.8 : Stimulation of purified T cells over time.
2 x 10^4 purified T cells were cultured with 2 x 10^4 transfectants (CHO or CD80 or CD58) in the presence of OKT3 (1μg/ml). Proliferation (a) and IL-2 production (b) were measured every 24 hours. Proliferation was measured using ^3H thymidine incorporation and IL-2 production using a CTLL bioassay. The data are from triplicate samples from a single representative experiment (n = 3).

Although the CD80 transfectants were able to stimulate purified T cells in the presence of OKT3 it was important to demonstrate that the costimulatory signal supplied to CD28 was due to ligation by the CD80 transfectant. This was investigated by the addition of CTLA-4-Ig to the assay. CTLA-4-Ig is a chimeric molecule, composed of the extracellular domain of CTLA-4 (which includes the CD80 binding site) fused to the constant region of an IgG antibody, which can bind CD80 with high affinity. The results in Figure 3.9 clearly show that the costimulatory function of the CD80 transfectant was disrupted by the addition of CTLA-4-Ig at concentrations as low as 500ng/ml. This demonstrated that CD80 binding of CD28 could be inhibited by the higher affinity ligand CTLA-4 and that proliferation was dependent on CD80 costimulation. It was not possible to examine the specificity of the CD80-CD28 interaction as a blocking agent for CD28 was unavailable. This could be investigated by using either Fab fragments of anti-CD28 mAbs or a chimeric CD28-Ig molecule and including either of these in the costimulation assay.

Effect of delaying the costimulatory signal.

In all the experiments described so far, costimulatory signals were provided by the transfectants at the same time as the initial first signal through the TCR/CD3 complex (using OKT3). To determine how long after an initial signal (via the TCR/CD3 complex) T cells were responsive to costimulation the effect of delaying this costimulatory signal was examined.

The results shown in Figure 3.10 demonstrated that if the second signal provided by CD80 transfectants was delivered more than 6 hours after the primary signal the T cells were unable to respond to the costimulation. This lack of responsiveness by the T cells was not due to loss of function by the fixed CD80 transfectants as these transfectants could stimulate freshly purified T cells to proliferate in the presence of OKT3 (Figure 3.11). This would suggest that the T cells were unable to respond to the costimulatory signals generated following ligation of CD28 by CD80.

The necessity of costimulation provided by CD28 has been demonstrated in a number of *in vitro* and *in vivo* systems where lack of costimulation results in T cell anergy or programmed cell death (Kawabe and Ochi, 1991; Tanaka et al., 1995; Nicolle et al., 1994; Shahinian et al., 1993; Mittrucker et al., 1996). As CD28 signalling has been shown to result in upregulation of IL-2 gene

transcription and stabilisation of IL-2 mRNA this suggests that lack of costimulation results in insufficient intracellular signals being induced, resulting in lack of IL-2 production - a necessary requirement for T cell proliferation. This has been demonstrated in previous research (Sansom et al., 1993) where the mitogen PHA was used to stimulate purified T cells. The T cells did not proliferate but were able to induce IL-2R surface expression. The T cells could be rescued by the addition of IL-2. It is possible that the T cells that were unresponsive to delayed signalling through CD28 (Figure 3.10) could also be rescued by addition of exogenous IL-2. However the unresponsive cells may not have been rendered anergic by TCR-only signalling but had been induced to undergo programmed cell death (apoptosis). As another role of CD28 signalling is to protect the T cells from apoptosis, (by upregulation of the bcl-$_{xl}$ gene which is known to protect cells from apoptosis) (Boise et al., 1995), lack of this protective signal may have induced apoptosis in the T cells.

There are a number of different methods to test which of these two hypotheses are correct. If the T cells are being induced to undergo apoptosis, viability of the T cells over time could be measured (by cell counting using the Trypan Blue exclusion method or by FACS analysis either by examining DNA content of the cells or by using propidium iodide which is taken up by dead cells). If the cells are viable and have possibly been anergised this could be tested by recovering the T cells and restimulating them using OKT3 and CD80 transfectants.

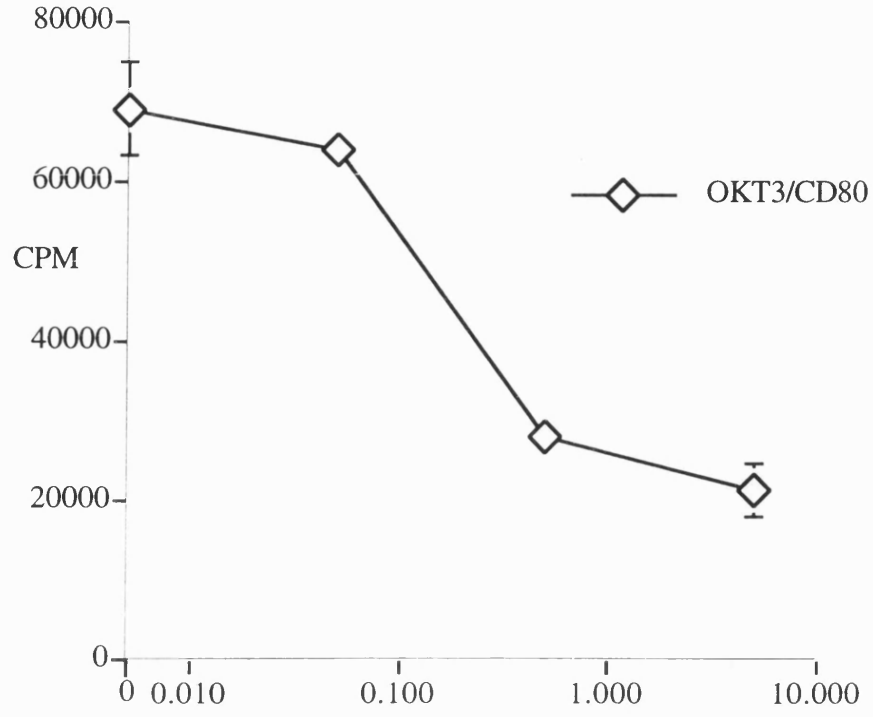


Figure 3.9 : Blocking the CD28 - CD80 interaction using CTLA4 -Ig.
5 x 10^4 purified T cells were cultured in the presence of OKT3 (1µg/ml) and 2 x 10^4 CD80 transfectants The chimeric molecule CTLA4-Ig (which binds CD80 with a higher affinity than CD28) was added at various concentrations (with a positive control being proliferation in the absence of CTLA4-Ig). Proliferation was measured using ^3H thymidine incorporation after 48 hours. The data are from triplicate samples from a single representative experiment (n = 3).

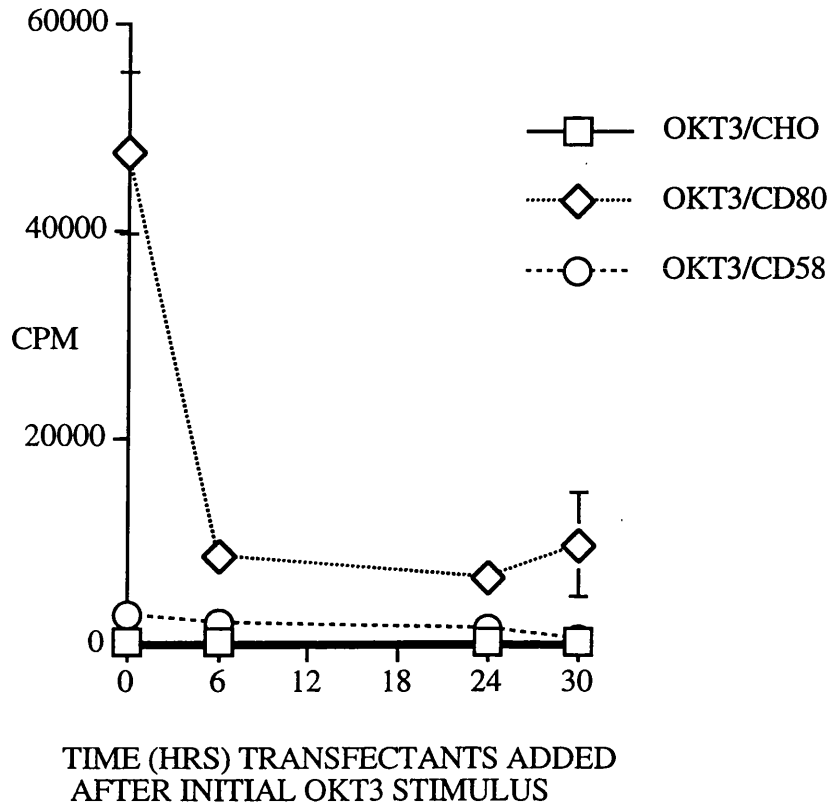


Figure 3.10 : Delay in providing costimulatory signal to purified T cells.
2 x 10^4 purified T cells were stimulated using OKT3 (1μg/ml) and 2 x 10^4 transfectants (CHO or CD80 or CD58) which were added at different times after the initial OKT3 stimulus. Proliferation was measured by 3H thymidine incorporation 48 hours after the transfectants were added. The data are from triplicate samples from a single representative experiment (n = 3).

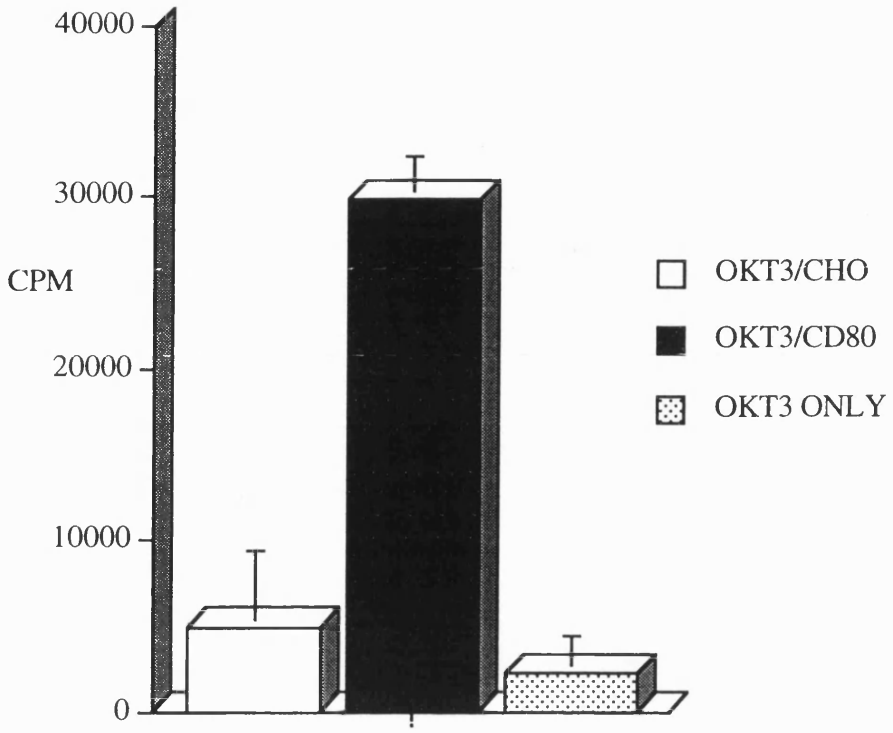


Figure 3.11 : The effect of fixation on CD80 transfectant function.
2 x 10^4 purified T cells were stimulated in the presence of OKT3 (1µg/ml) by 2 x 10^4 transfectants (either CHO or CD80) which had been fixed 7 days previously. Proliferation was measured after 72 hours by ^3H thymidine incorporation. The data are from triplicate samples from a single representative experiment (n = 3).

Effect of costimulation on CD28 surface expression.

The data generated so far have demonstrated the requirement for CD28 costimulation to activate T cells resulting in proliferation and IL-2 production. This costimulatory signal has also been shown to be time dependent i.e. CD28 signalling must occur within a few hours of TCR signalling to be effective in generating T cell proliferation. Since CD28 plays such an essential role in T cell activation it was important to examine whether ligation of CD28 by CD80 had any effect on the expression of CD28 itself.

Studies using polyclonal activators of T cells had already shown that CD28 expression was enhanced following T cell activation (Turka et al., 1990). To determine whether CD28 expression was similarly enhanced following T cell activation using OKT3 and CD80 transfectants, T cells cultured with OKT3 and transfectants were stained with mAbs to CD28 and surface expression of CD28 determined by FACS analysis.

Interestingly, the FACS analysis in Figure 3.12a shows that the surface expression of CD28 on T cells decreased within 24 hours of ligation by CD80 in the presence of OKT3. However CD28 surface expression slowly returned over a number of days towards its original unligated level. If no ligation of CD28 occurred, as when CHO cells were used in place of CD80 transfectants, there was no loss of CD28 from the T cell surface (Figure 3.12b). It would appear that ligation of CD28 by its ligand CD80 results in a decline in surface expression which is eventually reversed. However when T cells were stimulated by CD80 transfectants only (Figure 3.12c) the surface expression of CD28 also declined but on this occasion re-expression did not occur. This suggests that ligation of CD28 by CD80 is sufficient for downregulation of CD28 surface expression but that additional factors are required for re-expression of CD28. To ensure that these changes in the level of surface expression of CD28 were not due to variations in the machine set-up, the FACS was calibrated using standard fluorescent beads to the same level of intensity on each occasion as shown in Figure 3.13.

The data from figure 3.12c suggest that downregulation of CD28 surface expression occurred in response to CD80 ligation but re-expression of CD28 required additional factors. It has already been demonstrated that T cells stimulated by CD80 transfectants only (Figure 3.7) were unable to proliferate whereas T cells stimulated by OKT3 and CD80 transfectants could

proliferate (Figure 3.6). As the T cells stimulated with OKT3 and CD80 transfectants were able to re-express CD28 whereas the CD80 only stimulated T cells did not re-express CD28, this suggests that re-expression of CD28 may be linked to T cell activation.

Downregulation of CD28 surface expression following CD80 ligation has been previously reported (Linsley et al., 1993). In contrast to the results seen in figure 3.12b, the T cells in this study showed a much faster rate of downregulation and re-expression of CD28 - i.e. CD28 expression was downregulated within 12- 24 hours of ligation by CD80 and was re-expressed at its initial level or higher by 48 hours. However the T cells used by Linsley et al. had been stimulated using either PMA or PHA prior to ligation of CD28 by CD80 transfectants. It is possible that the T cells would be rapidly dividing in response to PMA or PHA whereas the T cells used in Figure 3.12 were unstimulated T cells and as has already been shown (Figure 3.8) T cell proliferation does not reach a maximum until 5 days after initial stimulation. It is possible that the difference in the kinetics of CD28 re-expression is due to differences in the level of cell cycling of the T cells used in these studies. As T cells stimulated by CD80 transfectants only are unable to proliferate and re-expression of CD28 on T cell stimulated by CD80 transfectants only did not occur (Figure 3.12c) this suggests the idea that CD28 surface expression is in some way responsive to proliferative signals.

Interestingly, Linsley et al. (1993) also found that the T cells following re-expression of CD28 had impaired CD28 signalling. This would suggest that although CD28 may be re-expressed at higher levels in activated T cells, signalling through CD28 would be ineffective. However, it is important to note that the T cells used to examine CD28 signalling by Linsley et al. had been stimulated twice - initially by PMA or PHA and then through CD28 following ligation by CD80 transfectants. It is possible that the hyporesponsiveness of the T cells is a method of preventing repeated restimulation of already activated T cells. Although T cells which have been activated can be restimulated further by ligation of CD28 (see Chapter 4) to enhance and prolong the immune response, restimulation of already activated T cells could not be allowed to occur repeatedly. The immune response would have to be controlled to allow it to subside when no longer required. This may occur by preventing further restimulation of the T cells due to CD28 hyporesponsiveness (as seen by Linsley et al.,1993) or by the generation of negative signals to switch off T cells.

Negative signalling may be a function of CTLA-4 which is upregulated on T cell activation and has been shown to generate intracellular signals following ligation which results in blocking IL-2 production (Krummel and Allison, 1996). Studies have suggested that CTLA-4 upregulation may be CD28-dependant - anti-CD28 mAbs were able to accelerate the kinetics of CTLA-4 mRNA in human peripheral blood lymphocytes and the level of CTLA-4 upregulation was significantly reduced on T cells from CD28-deficient mice (Lindsten et al., 1993; Walunas et al., 1994). More recent studies have shown that blockading CD28 ligation inhibits CTLA-4 upregulation (Walunas et al., 1996). These results suggest that a consequence of CD28 ligation is the surface expression of CTLA-4. As CTLA-4 has a higher affinity for CD80 than CD28, CTLA-4 would be expected to bind CD80 preferentially when T cells were restimulated. It may be the negative signals generated following CTLA-4 ligation predominate and override any positive signals due to CD28 ligation. In this way the immune response could be allowed to subside.

So far all these experiments have examined the role of CD28 in activating previously unstimulated T cells. However the surface expression of CD28 has been shown to be enhanced in activated T cells, stimulated by polyclonal activators (Turka et al., 1990, Linsley et al., 1993) and this increased surface expression of CD28 can also be seen on activated T cells even in the absence of CD80 ligation (Figure 4.3). Similarly, FACS analysis of T cell blasts generated using SEB has shown that the activated cells are larger and express higher levels of CD28 (McLeod et al., submitted). This increased level of expression of CD28 in activated T cells suggests that stimulation of CD28 may play an important role in augmentation of the immune response. As the CD80 transfectants had been able to ligate to CD28 on unstimulated T cells resulting in T cell activation (in conjunction with OKT3), these transfectants could be used to examine whether CD28 signalling following ligation of CD80 had any effect on already activated T cells.

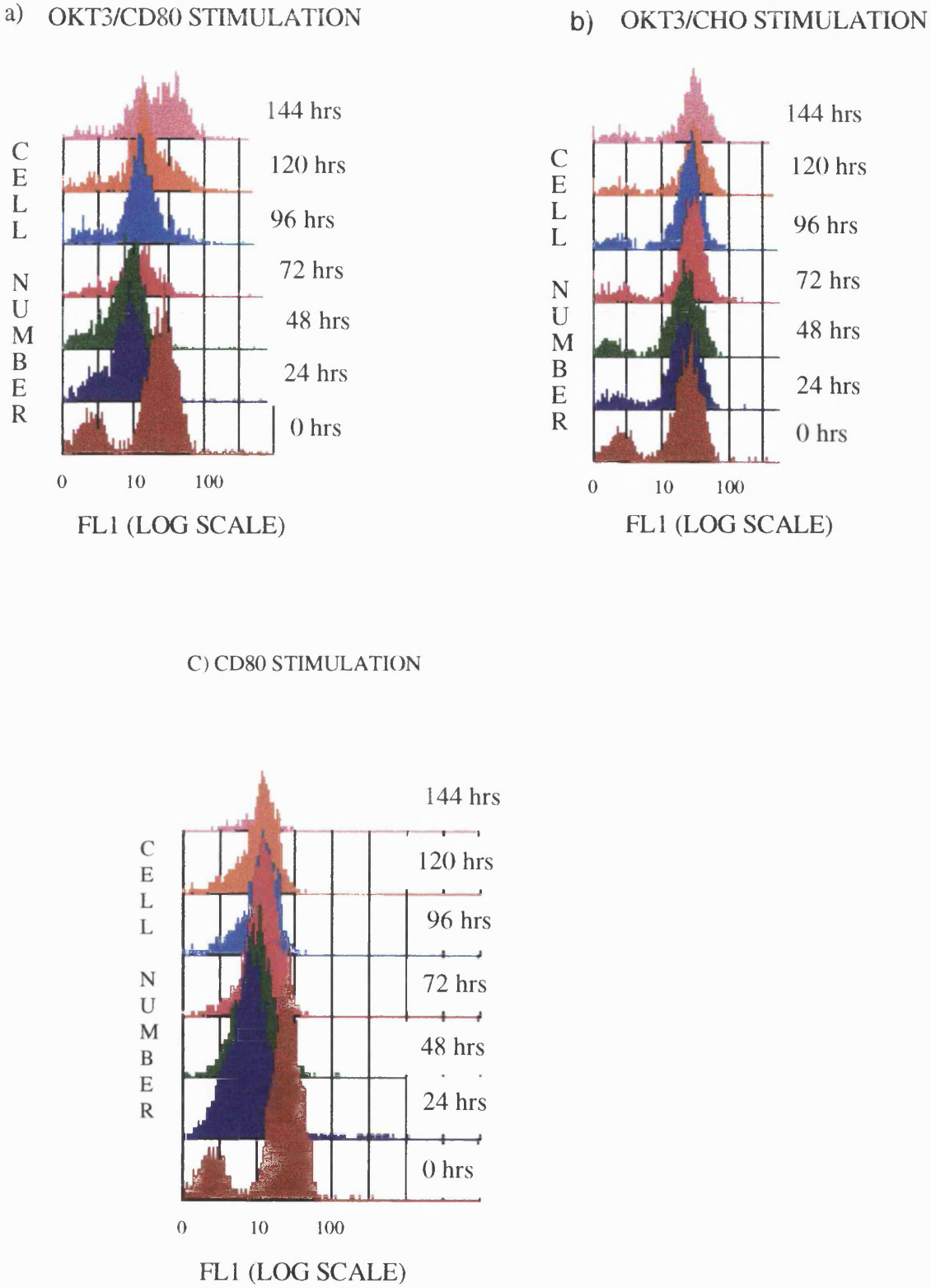


Figure 3.12 Surface expression of CD28 on purified T cells following stimulation by OKT3 and CD80

6 x 10^5 T cells were stimulated with OKT3 and 3 x 10^5 transfectants (CHO or CD80 transfectants) or CD80 transfectants only. The cells were removed at 24 hourly intervals and stained with 9.3 (anti-CD28 mAb). The surface expression of CD28 on the T cells was determined by FACS analysis.
The data are representative of a single experiment (n = 3).

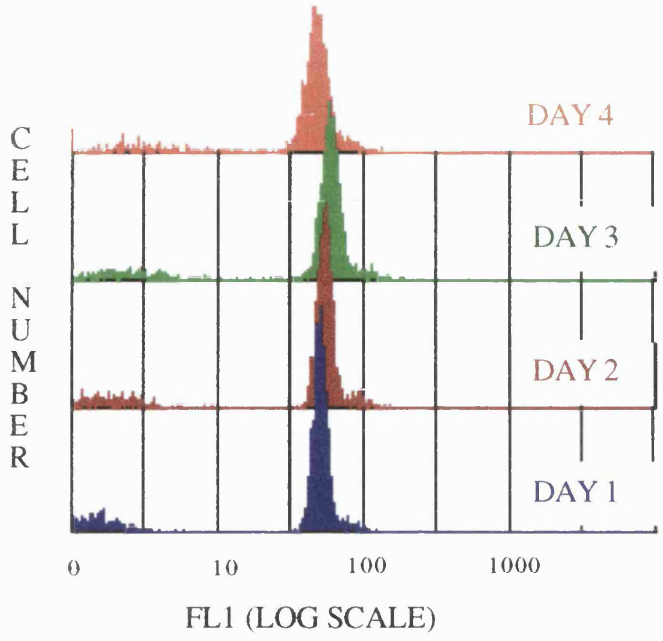


Figure 3.13. Calibration of Becton Dickinson FACStar Plus.
Standard fluorescent beads were analysed on the FACS and the laser calibrated to ensure that the same intensity of fluorescence (FL1) was obtained on all occasions.The data are representative of a single experiment (n>10).

Chapter 4.

CD28 stimulation in T cell blasts

Effect of stimulating CD28 on T cell blast proliferation.

Previous studies have shown that both PHA activated T cell blasts and SEB activated T cell blasts were responsive to stimulation through CD28 (Ledbetter and Linsley, 1992; Linsley et al., 1993; Sansom et al., 1993). In order to investigate the role of CD28 in stimulating activated T cells, T cell blasts were produced by stimulating PBMCs with a number of mitogens - SEB (1μg/ml) or PHA (10μg/ml) or PMA (50ng/ml) plus Ionomycin (1μM).

The resulting T cell blasts were washed and cultured in the presence of transfectants 4 days after their initial mitogenic stimulation. All three types of blasts showed an increase over background proliferation levels (using untransfected CHO cells as a control) when CD80 transfectants were present but not when CD58 transfectants were used (Figure 4.1). Under these conditions CD28 was able to respond to ligation by CD80 and the proliferative response of the T cell blasts was augmented. This was in contrast to unstimulated T cells which were unable to respond to CD80 transfectants alone.

Although all the T cell blasts generated could respond to CD80 stimulatory signals PMA/Ionomycin blasts were chosen for further experiments as the mitogenic stimuli could be removed by washing and it was possible to culture them in the absence of exogenous IL-2 for longer than either the SEB or PHA blasts.

The time dependency of stimulation of T cell blasts was examined by stimulating T cell blasts 4, 6, 8 and 10 days after initial mitogenic stimulation with CD80 transfectants. This revealed that proliferation of T cell blasts proliferation appeared to be time dependent as CD80 could only stimulate the blasts between days 4 - 8 post initial mitogenic signalling (Figure 4.2) - after that time the effect of CD80 was limited. This low response by the T cell blasts was not due to changes in the level of expression of CD28 as FACS analysis showed (Figure 4.3) that CD28 was always expressed on the surface of the T cells and was indeed upregulated with time. The method of producing T cell blasts did not result in downregulation of CD28 expression even at the initial stages of stimulation unlike stimulation of resting T cells using OKT3 and CD80 transfectants. This was due to PMA and Ionomycin stimulating the T cells via components of the T cell signalling pathway (stimulation of PKC and extracellular Ca^{++} influx respectively) and not involving CD28 ligation. It

may be that the lack of responsiveness by T cell blasts to further costimulation after 8 days represents a form of control by the T cells to ensure that an immune response can be enhanced in the early stages but not indefinitely so that the immune response may subside when no longer required.

The activated T cell blasts stimulated by the CHO cells show high levels of proliferation at day 4 but this level of proliferation declines rapidly over time (Figure 4.2). As the T cell blasts are able to proliferate without any further stimulation being supplied, this would suggest that some active signalling pathways are present in the T cells. However the decline in T cell proliferation with time in the absence of other stimuli suggests that these activation pathways may decay and proliferation as a result declines. The ability of CD80 to augment proliferation in activated T cells suggests that signalling through CD28 may synergise with the active signals present in activated T cells resulting in increased proliferation levels. The lack of augmented proliferation in older T cell blasts despite CD80 stimulation may be due to the signalling pathways in the T cells decaying to a point where the T cells are unable to respond to the CD28 signals generated.

Although CD28 signalling has been shown to augment proliferation of activated T cells, it is important to realise that this stimulation of already activated T cells is also a method of sustaining the immune response. The importance of CD28 in sustaining T cell responses has been demonstrated *in vivo* in CD28 knockout mice where murine T cells were able to respond to mitogen stimulation but this response could not be sustained (Lucas et al., 1995). CD28 may play a major part in ensuring that T cells activated in response to foreign antigen can survive to ensure that the immune response generated does not quiesce too soon. However the activated T cells cannot be allowed to be continually sustained and lack of response of older T cell blasts to CD28 signalling may be a controlling mechanism.

However it should also be remembered that activation of T cells results in upregulation of CTLA-4 on the T cells which is known to provide negative signals to the T cells following ligation by CD80 (Krummel and Allison 1996, Walunas et al., 1996). The lack of responsiveness of older T cell blasts may be due to the increasing role CTLA-4 plays in cell signalling.

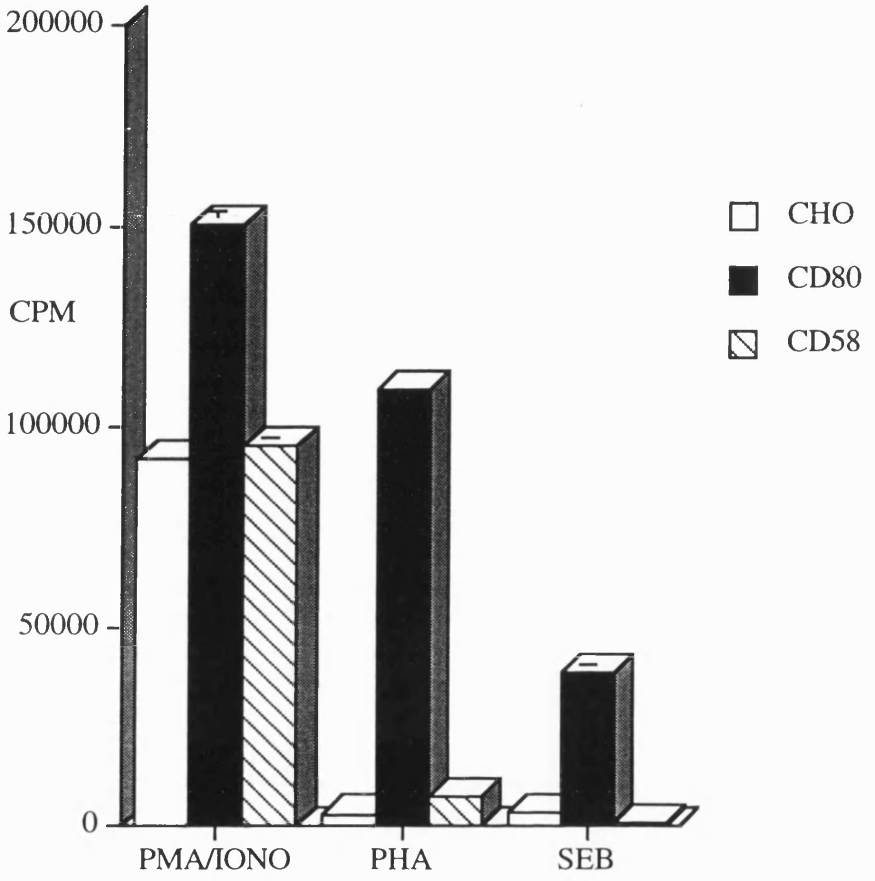


Figure 4.1 Stimulation of T cell blasts by CD80.
T cell blasts (5 x 10^4) 4 days after initial stimulation (with either PMA and Ionomycin or PHA or SEB) were cultured with transfectants (2 x 10^4) as shown. Proliferation was measured after 48 hours using ^3H thymidine incorporation. The data are from triplicate samples from a single representative experiment (n = 3).

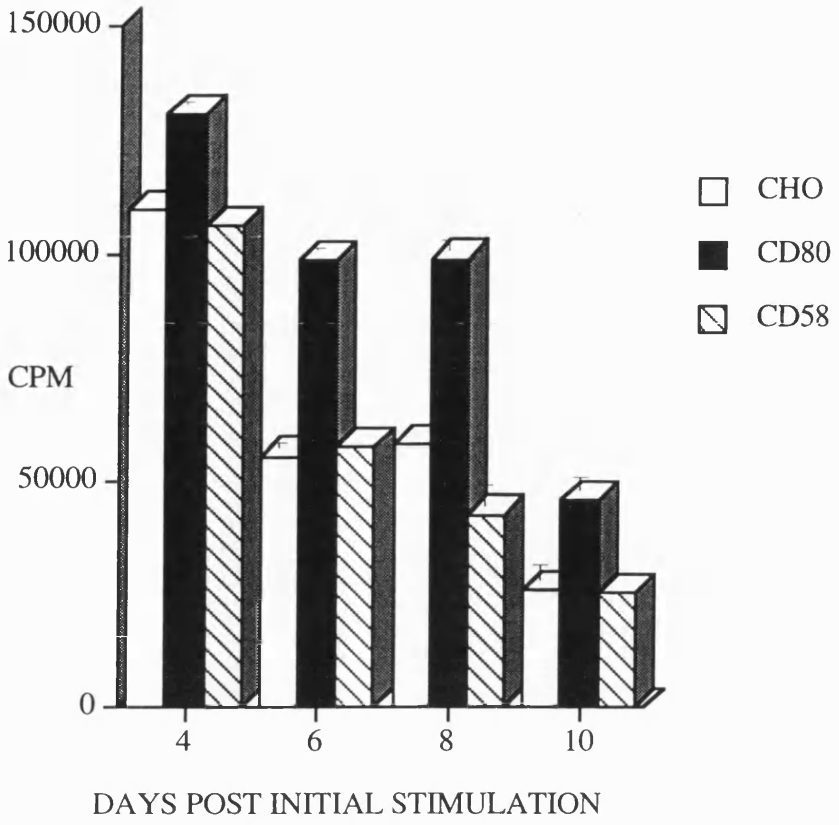


Figure 4.2 CD80 augmentation of T cell blast proliferation over time.
2 x 10^4 PMA/Ionomycin T cell blasts at 4, 6, 8 and 10 days after their initial stimulation were cultured with 5 x 10^3 transfectants as shown. Proliferation was measured after 48 hours using ^3H thymidine incorporation.The data are from triplicate samples from a single representative experiment (n = 3). Additional data is tabulated in appendix 5.

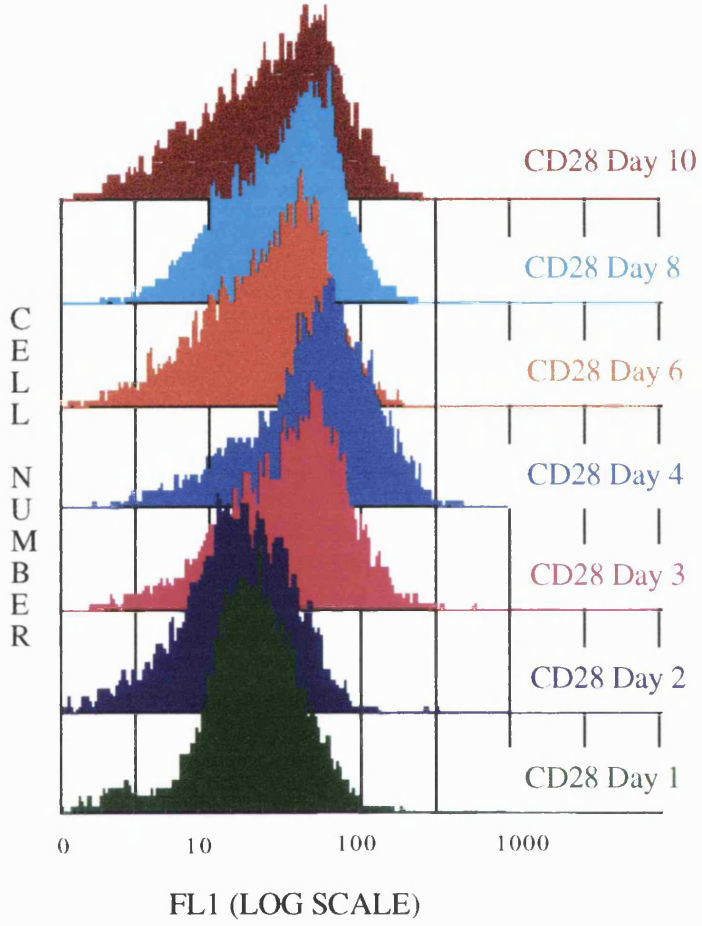


Figure 4.3 Surface expression of CD28 on T cell blasts over time.
PMA and Ionomycin T cell blasts were stained on a daily basis using 9.3 (anti-CD28 mAb) and the surface expression of CD28 on the T cell blasts determined by FACS analysis. The data are representative of a single experiment (n=5).

IL-2 production by T cell blasts stimulated through CD28.

As the CD80 transfectants were able to augment the proliferation of T cell blasts and a major feature of CD28 costimulation of purified T cells is increased IL-2 production, it was important to determine whether IL-2 production in T cell blasts was affected by CD28 stimulation. T cell blasts were cultured with either CD80 transfectants, CD58 transfectants or the CHO parental cell line at 4, 6, 8 and 10 days after the initial PMA/Ionomycin stimulation. The proliferation (Figure 4.2) demonstrated that only CD80 was able to augment the proliferation of the already activated T cell blasts.

In striking contrast to the proliferation data, IL-2 was only produced at significant levels when using T cell blasts 4 days after their initial stimulation Figure 4.4). However if older T cell blasts were used in the experiment (i.e. 6, 8 or 10 days after initial stimulation) although proliferation was enhanced by culturing in the presence of CD80 transfectants there was negligible production of IL-2 either by stimulated or unstimulated T cell blasts.

These results were surprising since it may suggest that IL-2 is not essential for some forms of T cell proliferation. However as has already been stated in chapter 3, the lack of detection of IL-2 may be due to the poor sensitivity of the bioassay used as only unbound IL-2 is measured. If the rate of IL-2 production increases but at the same time the turnover rate of IL-2 also increases, the increase in IL-2 production levels would not be detected by the bioassay as there would be no increase in the levels of unbound IL-2. To try and address this issue, cytokine production was measured by examining the transcription levels of mRNA for IL-2. If IL-2 was being produced at higher levels but the turnover rate of the protein was such that its presence could not be detected using a bioassay there should have been an increase in the level of mRNA produced. Since the increase in proliferation of T cell blasts in response to CD28 stimulation using CD80 transfectants was striking, if IL -2 production had increased and was responsible for generating this enhancement of T cell proliferation, the change in cytokine mRNA levels should be obvious. It was necessary to use RT-PCR (a method of amplifying mRNA) to detect levels of the mRNA of interest for two reasons - 1) the number of T cells available for use in each experiment was restricted by the amount of blood that could be donated and 2) the amount of RNA found in each cell is also limited (10pg/cell) of which only 1-5% is mRNA.

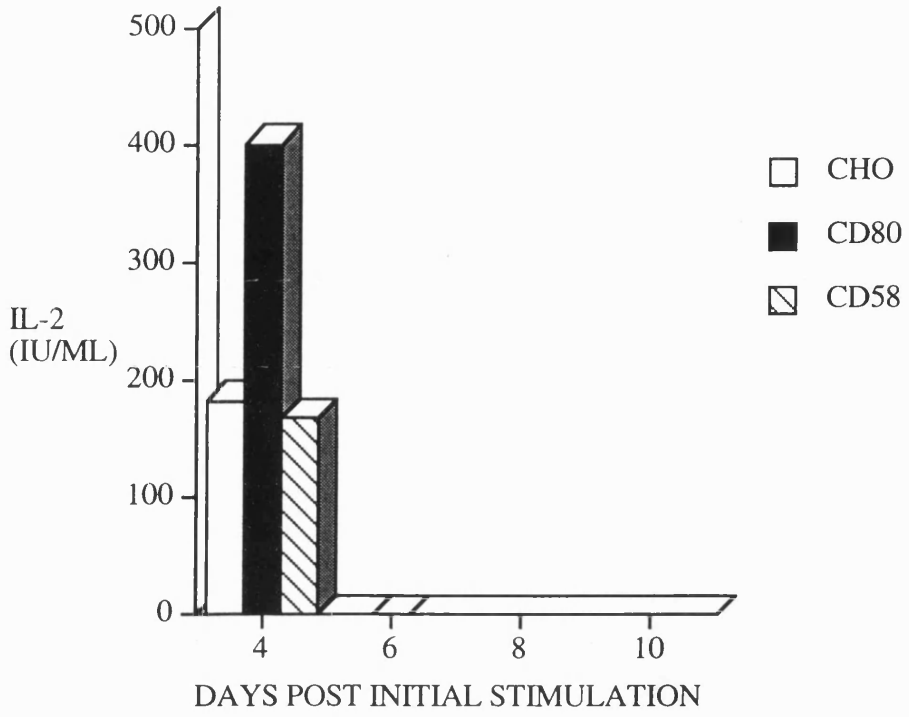


Figure 4.4 IL-2 production by T cell blasts.
2 x 10^4 T cell blasts 4, 6, 8 and 10 days post initial stimulation were cultured with 5 x 10^3 transfectants (as shown). IL-2 production was measured after 48 hours using a CTLL bioassay respectively. The data are from triplicate samples from a single representative experiment (n = 3). Additional data is tabulated in appendix 5.

Before determining whether the IL-2 mRNA levels of T cell blasts were increased by CD80 costimulation it was necessary to establish a reliable RT-PCR method. The RNA collected from the cells in each experiment was run out on a 1% agarose gel to ensure it had not degraded during its extraction. Figure 4.5 shows a typical RNA gel with the bands of ribosomal RNA (28s, 18s and 6s) being visible. The next parameter to be determined was how soon after stimulation IL-2 mRNA was produced. PBMCs were stimulated with PMA and Ionomycin as before and RNA extracted either before stimulation or 1, 3 or 5 hours after stimulation. The results of RT-PCR using primers for IL-2 gene are shown in figure 4.6a. The IL-2 mRNA was not detected in unstimulated PBMCs (lane 4) but was present as soon as 1 hour after stimulation of the PBMCs with PMA and Ionomycin. The lack of IL-2 mRNA in unstimulated PBMCs was not due to degradation of the RNA as not only was the RNA checked prior to the RT-PCR being performed but primers for the GAPDH housekeeping gene were able to amplify cDNA from the unstimulated PBMCs (Figure 4.6b Lane 4). As there appeared to be little difference in the amount of IL-2 mRNA detected after 1, 3 or 5 hours it was decided to use 3 hours as a standard stimulation time before collecting RNA samples.

As T cell blasts were being cultured with fixed transfectants it was important to determine whether RNA could be extracted from the transfectants. Figure 4.5 lane 1 clearly shows RNA from CD80 transfectants. However, when RNA from fixed transfectants was used in RT-PCR using primers for IL-2 and GAPDH only the GAPDH mRNA was detected (Figure 4.7 lane 3), IL-2 RNA transcripts were not present (lane 2). This suggested that although the mRNA from the fixed transfectants would be extracted along with that from the T cell blasts, the lack of mRNA for IL-2 in the transfectants would ensure that any IL-2 mRNA amplified using RT-PCR would have originated in the T cell blasts.

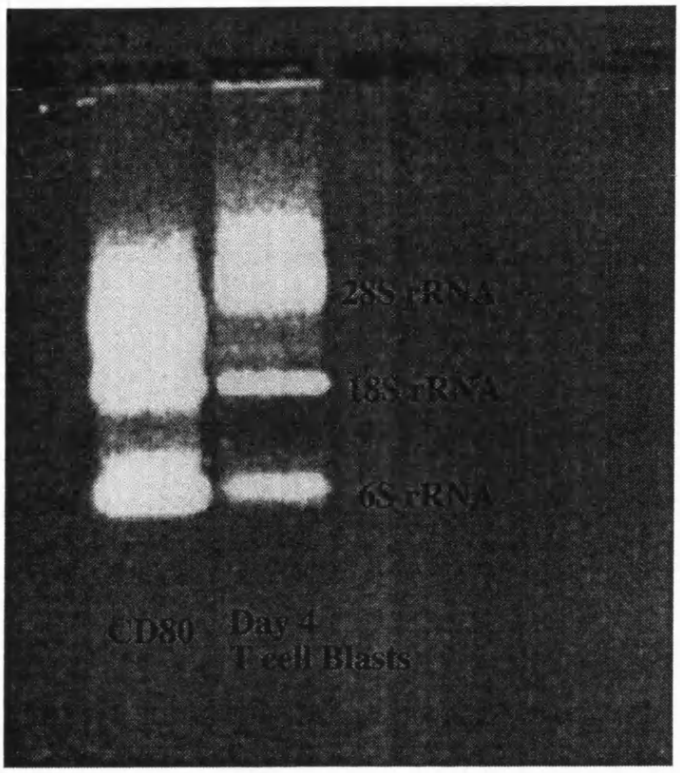


Figure 4.5. Evaluation of RNA extraction from T cell blasts and transfectants. Approximately 1μg of RNA extracted from T cell blasts and transfectants was evaluated on a 1% agarose gel by electrophoresis. The gel contained ethidium bromide which intercalates between the bases of the RNA and is fluorescent in UV light making the RNA visible. The bands in both lane 1 (RNA from CD80 transfectants) and lane 2 (RNA from T cell blasts) correspond to ribosomal RNA (28s, 18s and 6s).There is no large accumulation of RNA at the bottom of the gel indicating that the RNA had not degraded during the extraction process.

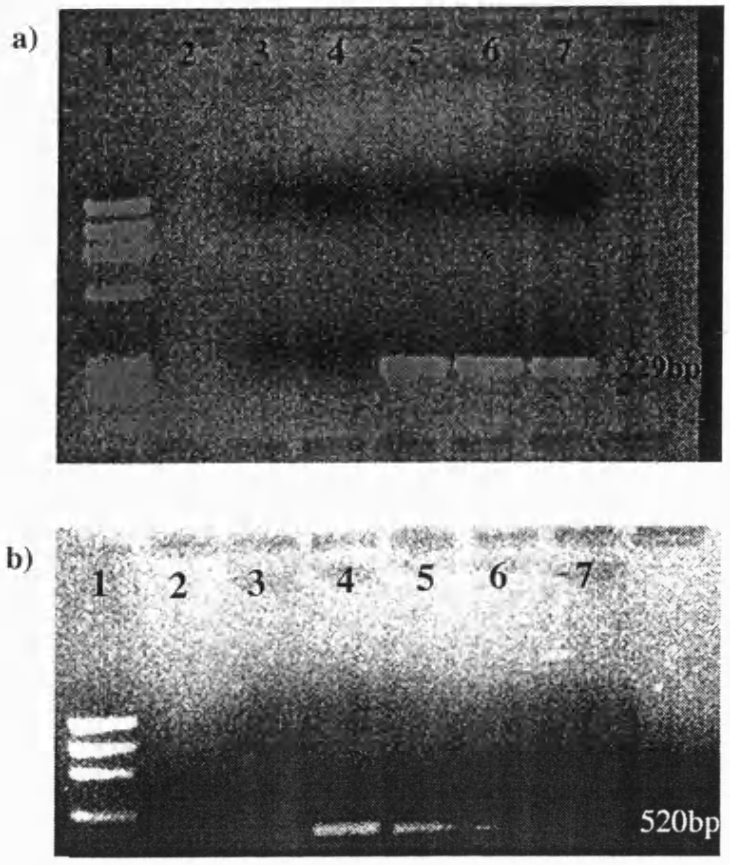


Figure 4.6 IL-2 transcription in stimulated PBMCs.
5 x 10^5 PBMCs were stimulated with PMA (50ng/ml) and Ionomycin (1μM) and the RNA extracted 1, 3 and 5 hours post stimulation. RNA was also extracted from unstimulated PBMCs. The RNA was reverse transcribed and the cDNA produced was amplified using primers to the IL-2 and the GAPDH genes. The amplified DNA was separated on a 2% agarose gel by electrophoresis. The marker ϕX 174 DNA digested with Hae lll was also run to indicate to size of the individual bands. Figure 4.6a shows IL-2 transcripts Figure 4.6b -GAPDH transcripts. Lane 1 contains the marker. Lane 4 unstimulated PBMCs. The RNA extracted, reverse transcribed and cDNA amplified from stimulated PBMCs is shown in lane 5 (1 hour post stimulation) lane 6 (3 hours post stimulation) and Lane 7 (5 hours post stimulation).

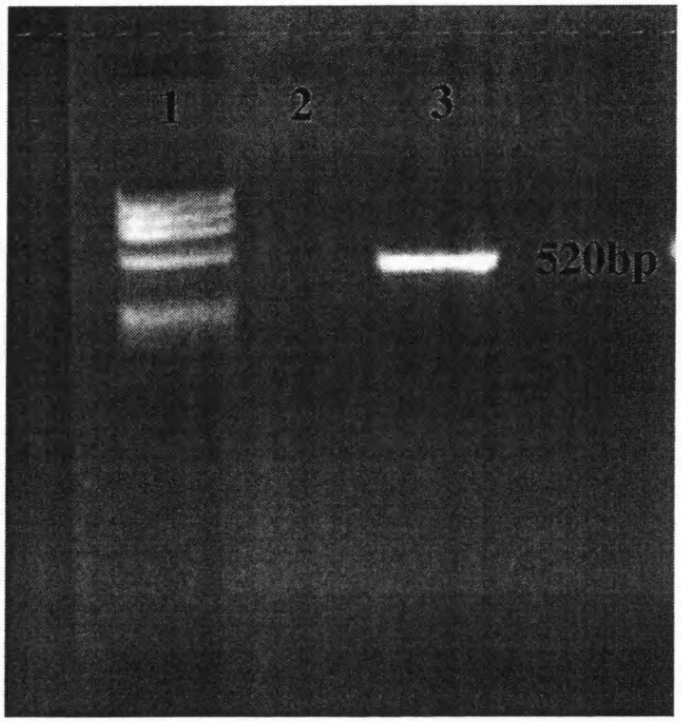


Figure 4.7 RNA from transfectants did not include IL-2 mRNA.
RNA from CD80 transfectants was extracted and reverse transcribed. the cDNA obtained was amplified by PCR using primers for the IL-2 and GAPDH genes. The resulting DNA was evaluated on a 2% agarose gel. The marker ϕX 174 DNA digested with Hae lll was also run to indicate to size of the individual bands. The marker is in lane 1. The DNA produced by amplification using the IL-2 primers is shown in lane 2 whereas the DNA produced using the GAPDH primers is in lane 3.

Having established that RT-PCR could be used to detect mRNA for IL-2 in PBMCs and that although fixed transfectants did contain mRNA this did not include IL-2 mRNA, this technique was used to examine IL-2 mRNA in T cell blasts. T cell blasts were cultured with either CD80 transfectants or CHO parental cells for 3 hours and their RNA extracted. RT-PCR showed (Figure 4.8a) there were no substantial differences in the level of IL-2 mRNA present in the T cell blasts comparable to that seen in Figure 4.6. If the level of the mRNA from GAPDH is examined (Figure 4.8b) similar levels were found in all T cell blasts regardless of age and stimulation confirming that the lack of substantial variation in IL-2 levels was not due to discrepancies in the RT-PCR method.

The lack of tangible variation in IL-2 mRNA levels in T cell blasts was unexpected since proliferation in response to CD28 signalling in T cell blasts increased and a major feature of CD28 signalling in resting T cells is to increase IL-2 mRNA transcription and increase its stability. However, the ability of CD28 to generate signals resulting in increased proliferation of T cell blast in the absence of IL-2 production suggests that a different signalling pathway was utilised in T cell blasts. This will be discussed in Chapter 6.

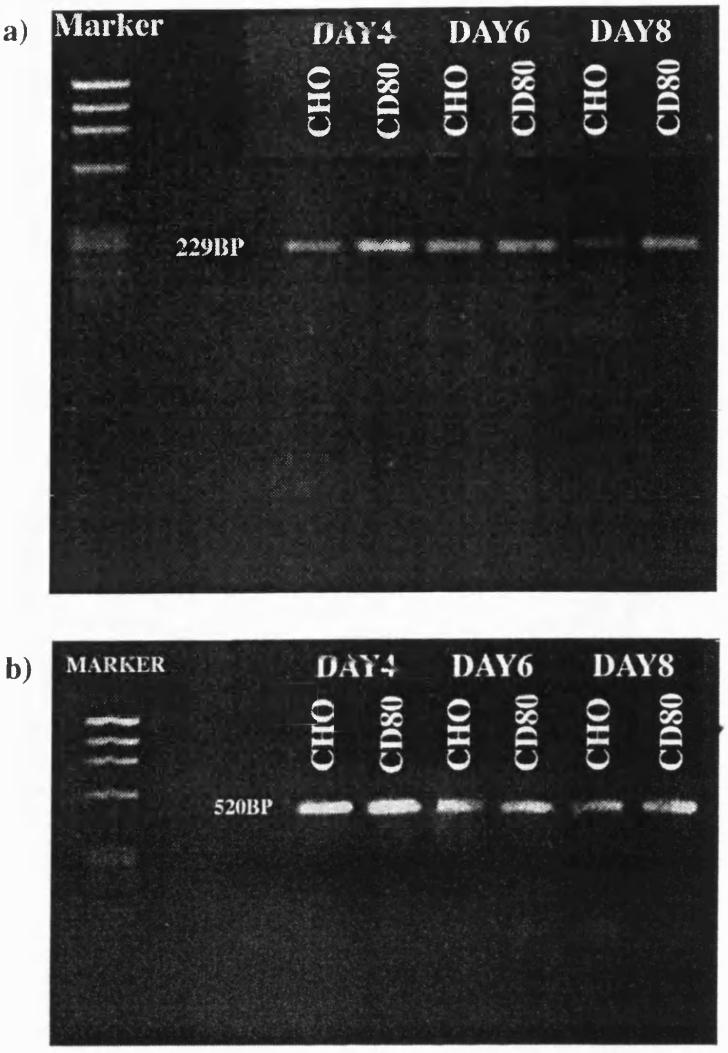


Figure 4.8: IL-2 transcription from T cell blasts stimulated by transfectants. T cell blasts were produced (as detailed in figure 4.6) and 5 x 10^5 blasts (4, 6 or 8 days post stimulation) were cultured with 1 x 10^5 transfectants (CHO or CD80) and the RNA extracted after 3 hours. The RNA was reverse transcribed and amplified by PCR using IL-2 or GAPDH primers. The resulting DNA was evaluated on a 2% agarose gel by electrophoresis. The marker ϕX 174 DNA digested with Hae lll was also run to indicate to size of the individual bands.
Figure 4.8a shows IL-2 transcription
Figure 4.8b shows GAPDH transcription

The increase in T cell proliferation by activated T cell blasts following stimulation of CD28 in the absence of IL-2 production highlights the multiple roles CD28 signalling has in T cell activation. Although the most obvious feature of CD28 signalling is the effect these signals have on IL-2 gene transcription (increasing transcription, stabilising IL-2 mRNA and induction of IL-2 transcription factors (Tonks et al., 1988; Fraser et al., 1991; Fraser et al., 1993; Edmead et al., 1996) other signals must be involved to sustain T cell proliferation in already activated T cells in the absence of IL-2. Previous research has demonstrated that CD28 costimulatory signalling is required to prevent induction of anergy or apoptosis in unstimulated T cells. The ability of CD28 to sustain the survival of T cells may be as a result of CD28 signalling upregulating the transcription of the bcl-xl gene which is known to protect cells from apoptosis (Boise et al., 1993). The evidence from CD28 knockout mice also suggest a protective role for CD28 as the T cells in these mice although able to respond to stimuli are not able to sustain an immune response (Lucas et al., 1995). Perhaps the lack of a protective signal by CD28 to the activated T cells in the knockout mice resulted in the induction of apoptosis.

The signals generated by CD28 following ligation by CD80 are complex and can result in the induction of IL-2 production which will activate unstimulated T cell and generate proliferation, as well as prolong an already established immune response in the absence of IL-2. In both these situations CD28 signalling results in the survival of the T cell - lack of CD28 signalling can result in apoptosis in previously unstimulated cells and prevent sustaining an immune response in activated T cells (Tanaka et al., 1995; Kawabe and Ochi, 1991; Nicolle et al., 1994; Lucas et al., 1995). One of the consequences of T cell activation is the upregulation of CTLA-4 and CD80 and the possible interactions between T cells bearing all three surface ligands (i.e. CD80, CD28 and CTLA-4) has still be fully investigated and understood.

Chapter 5

CD86 - a second ligand for CD28

Effect of CD86 on costimulation of purified T cells.

With the discovery of a second ligand for CD28 - CD86 (also known as B70 or B7-2) (Hathcock et al., 1993; Freeman et al., 1993; Azuma et al., 1993a), it was important to determine whether CD86 had a role to play in T cell activation. As described in Chapter 3, a CD86 transfectant was generated and cloned to produce a stable expressing cell line which could be used in the established test system to determine if there were any functional differences between the two CD28 ligands - CD80 and CD86.

To determine whether the CD86 transfectant could provide the necessary costimulatory signals to activate T cells, purified unstimulated T cells were cultured in the presence of OKT3 with CD80, CD86 transfectants and the untransfected parental cell line. CD86 transfectants were capable of providing costimulatory signals to purified T cells in conjunction with OKT3 resulting in IL-2 production and T cell proliferation (Figure 5.1). This demonstrated that CD86 ligation of CD28 could generate the necessary costimulatory signals to induce T cell proliferation and IL-2 production and CD86 could function as an alternative ligand for CD28 costimulation. However, although both CD80 and CD86 costimulation resulted in T cell proliferation the level of proliferation produced by CD86 transfectants was always greater than that produced by CD80 transfectants. The margin of this difference varied between individuals (Figure 5.2) but on all occasions proliferation in response to CD86 costimulation was higher. This variation in proliferation levels generated was not due to differences in surface expression of CD80 and CD86 on the CHO transfectants as FACS analysis of the cloned transfectants show similar levels of expression (Figure 5.3). The increased levels of proliferation generated when CD86 transfectants were providing costimulation was not due to greater affinity of the ligand for its receptor as CD86 has a similar receptor binding properties to CD80 (Linsley et al., 1994). The only major difference between CD80 and CD86 is in their distribution with CD86 being more widely expressed than CD80 which tends to be expressed later following activation. It would therefore seem likely that CD86 would be the ligand available to bind CD28 in unstimulated T cells and provide the necessary costimulatory signals i.e. CD86 may be the primary natural ligand for CD28. This difference between the efficiency of CD86 and CD80 transfectants to costimulate purified T cells has also been seen in research on antigen - specific mouse T cells (Hathcock et al., 1994). Using blocking mAbs to CD80 and CD86, proliferation of the antigen specific T cells in response to antigen presented by

mitomycin -treated APCs could only be inhibited by the addition of anti-CD86 mAbs and that addition of anti-CD80 mAbs did not result in increased inhibition. This suggested that CD86 was primarily involved in providing the necessary costimulation to the T cells. *In vivo* studies also suggest a dominant role for CD86 in the primary immune response as CD86 knockout mice were found to be severely immunocompromised whereas CD80 knockout mice were able to mount an effective humoral and cell mediated immune response (Borriello et al., 1997).

The research using antigen- specific mouse cells (Hathcock et al., 1994) also showed that blocking CD86 costimulation inhibited IL-2 production but not the induction of activation antigens - CD69 and IL-2R α chain. Blocking CD86 - CD28 interactions would result in inhibiting the CD28 signals which are involved in induction of the IL-2 gene - increasing IL-2 mRNA transcription, increasing the stability of the IL-2 mRNA and the induction of transcription factors for the IL-2 gene. The lack of IL-2 production suggests that the antigen specific signals generated by the TCR were insufficient to induce IL-2 production. This requirement for CD28 signalling has already been demonstrated (Chapter 3) where costimulatory signals provided by either CD58 transfectants or untransfected CHO cells were unable to induce IL-2 production or T cell proliferation despite TCR signalling in response to the mAb OKT3.

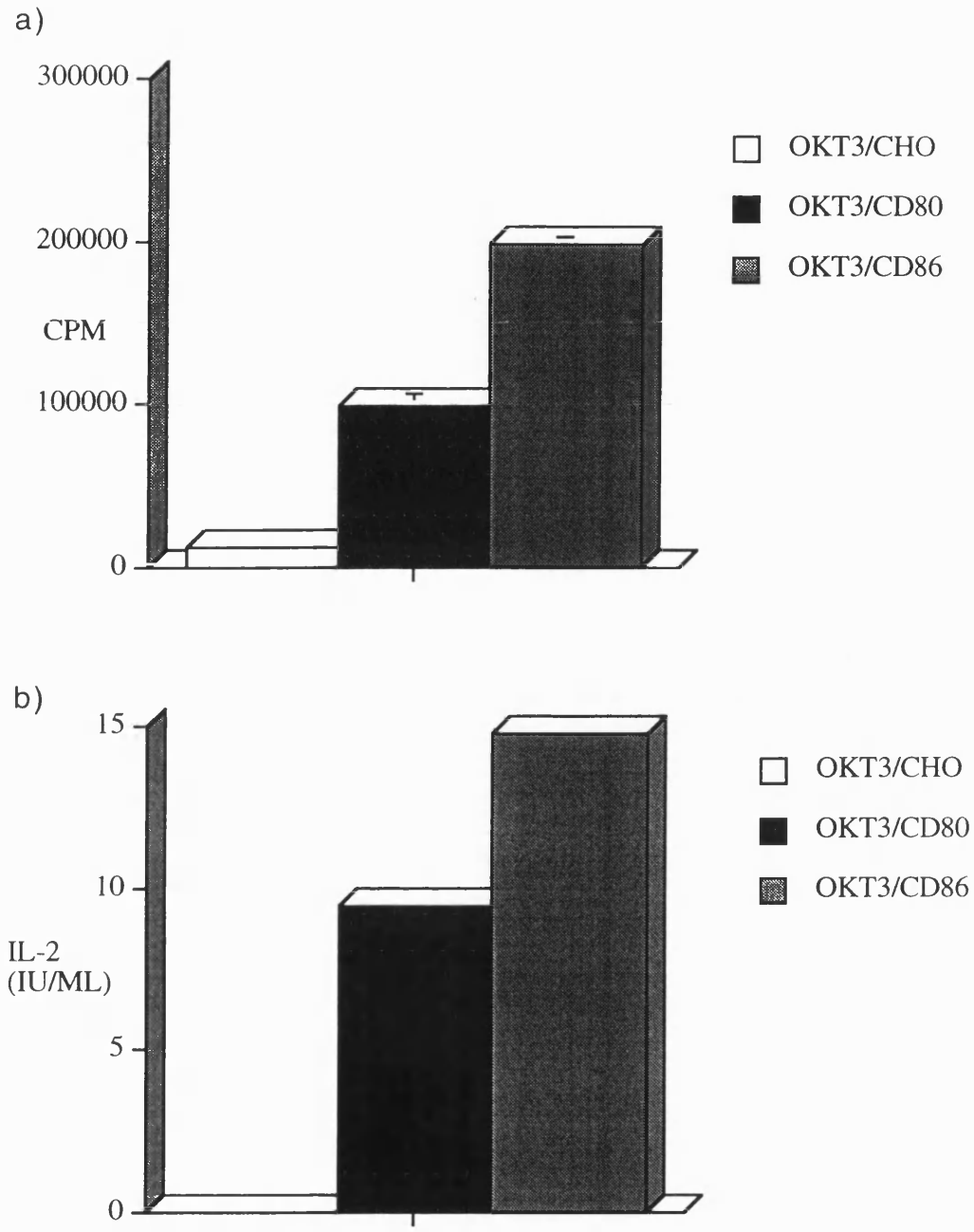


Figure 5.1 CD86 costimulation of purified T cells.
3 x 10^4 T cells were stimulated in the presence of OKT3 (1μg/ml) with 2 x 10^4 transfectants as shown. Proliferation and IL-2 production were measured after 48 hrs by ^3H thymidine incorporation and CTLL bioassay respectively. The data are from triplicate samples from a single representative experiment (n = 3).

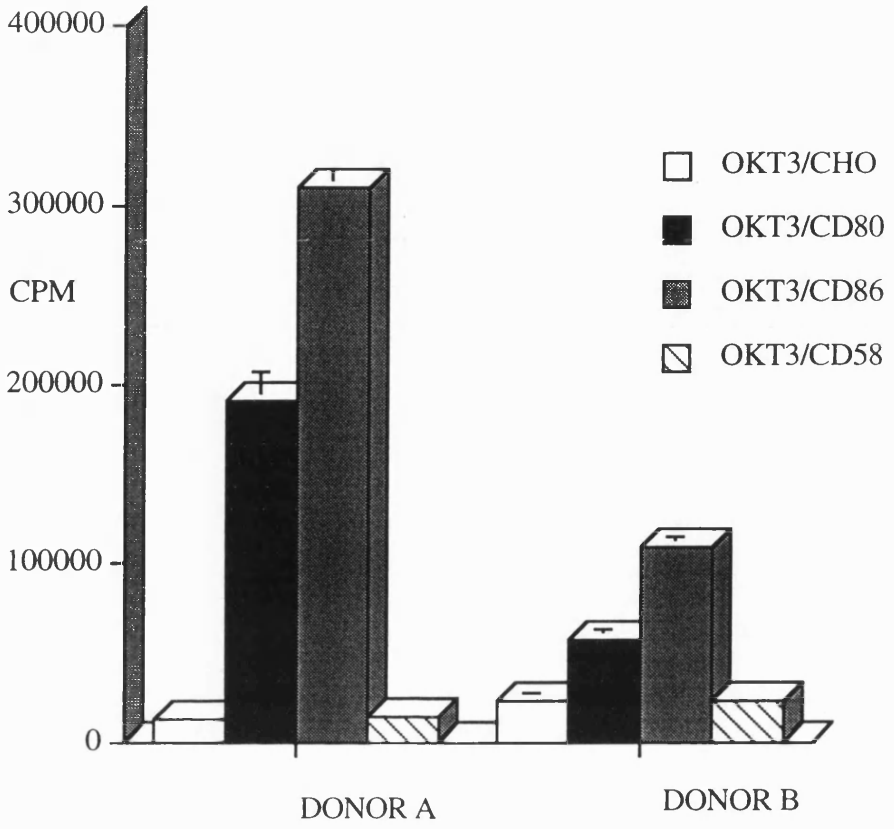


Figure 5.2 Comparison of CD86 and CD80 costimulation of purified T cells from different donors.
3 x 10^4 T cells were stimulated in the presence of OKT3 (1µg/ml) with 2 x 10^4 transfectants as shown. Proliferation was measured after 48 hrs by ^3H thymidine incorporation.

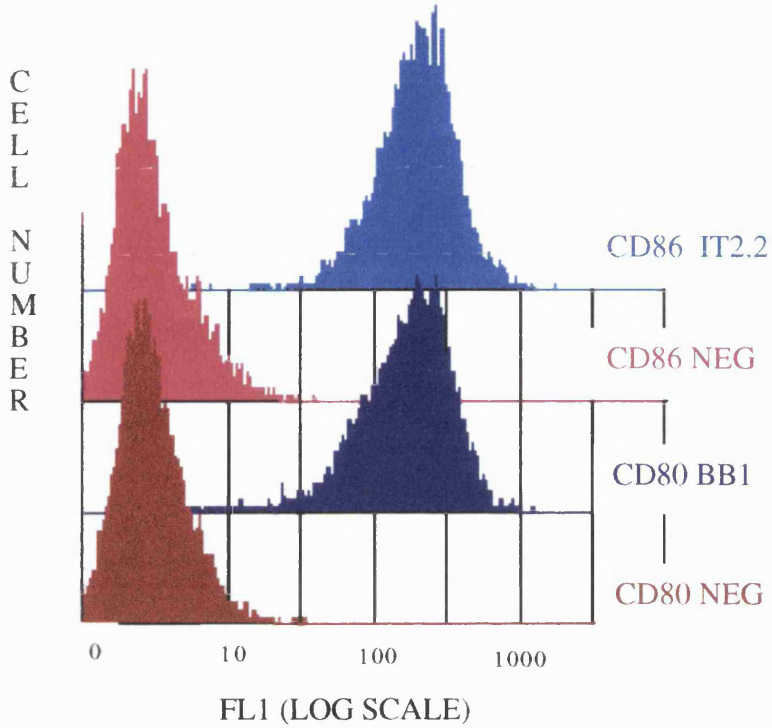


Figure 5.3 Comparison of surface expression levels of CD80 and CD86 on transfectants.

CD80 and CD86 transfectants were stained with their respective mAbs (BB1 for CD80 and IT2.2 for CD86). The surface expressions of CD80 and CD86 on the transfectants was determined by FACS analysis. The background level of fluorescence for each transfectant is also shown. The data are representative of a single experiment (n>10).

Effect of CD86 stimulation on activated T cells.

Having demonstrated that CD86 ligation could provide the necessary costimulatory signals to induce IL-2 production and T cell proliferation, it was important to assess if CD86 could stimulate previously activated T cells. The results shown in Figure 5.4 demonstrated that CD86 was indeed able to enhance T cell blast proliferation as had CD80. Similarly, this enhancement of proliferation was time dependent with the greatest effect being seen in day 6 and day 8 blasts. By day 10 there was limited stimulation by CD86. However, although CD86 was able to enhance proliferation of previously activated T cell blasts it did not appear as effective a stimulator as CD80. This was seen on every occasion but as with comparison of costimulation of unactivated T cells, the margin of the difference in proliferation generated varied depending on the individual T cell donor (Figure 5.5). This difference in enhanced proliferation between CD80 and CD86 was not due to variation in the surface expression of these molecules (see Figure 5.3). In fact the effect of stimulating T cell blasts with CD80 and CD86 transfectants was the reverse of that obtained when resting T cells were used where CD86 provided the greater costimulatory signals. It may be that as CD80 expression is upregulated on activation, CD80 rather than CD86 is the more natural ligand for CD28 on activated T cells. This suggests that CD86 is involved in induction of T cell proliferation, whereas CD80 may be responsible for sustaining T cell activation.

CD86 has been shown to stimulate T cell blasts although not as effectively as CD80. As a major feature of CD28 costimulation in unactivated T cells is increasing IL-2 production it was important to assess if CD86 stimulation of T cell blasts had any effect on IL-2 production. The results of stimulation of T cell blasts by CD86 on IL-2 production were consistent with those produced by CD80 stimulation of T cell blasts - i.e. high levels of IL-2 production were only detectable in Day 4 T cell blasts (figure 5.6). Older blasts although stimulated by both CD80 and CD86 (as seen by their increased proliferation levels) did not produce detectable levels of IL-2. As with CD80 stimulation of T cell blasts this lack of detection of IL -2 production by older blasts may be due to the limitations of the bioassay used which only measures unbound IL-2. To determine whether there was increased IL-2 production by older T cell blasts, mRNA from T cell blasts stimulated by CD86 was extracted and amplified using RT-PCR (Figure 5.7). As already seen for CD80 in chapter 4 although T cell proliferation was augmented by ligation of CD86 transfectants

there was no substantial increase in the level of IL-2 mRNA transcription detected in the T cell blasts. This lack of substantial variation in IL-2 transcription levels was not due to inconsistencies in the RT-PCR method used as the level of transcription of the housekeeping gene GAPDH was the same for all the T cell blasts regardless of their age or the cells they had been cultured with.

Thus, as with CD80 stimulation of T cell blasts, although CD86 was able to enhance proliferation of T cell blasts this did not appear to be due to increased IL-2 production as demonstrated by both the bioassay and the RT-PCR. This suggests that although CD86 ligation of CD28 was having an effect on T cell blast proliferation the signals generated by the interaction of CD28 and CD86 were not the same as those generated when purified unstimulated T cells were stimulated. A major function of CD28 signalling is to increase IL-2 mRNA transcription and increase its stability (Lindsten et al., 1989; Fraser et al., 1991). The IL-2 data from the bioassay and the RT-PCR suggests that IL-2 was not involved in increasing T cell blast proliferation in response to signalling through CD28 and that an alternative signalling pathway had been used resulting in increasing T cell blast proliferation. CD28 signalling will be discussed in Chapter 6.

These experiments have shown that CD86 can act as a ligand for CD28 and induce T cell proliferation in both unstimulated T cells (in conjunction with OKT3) and in T cell blasts. The signals generated by CD28 in unstimulated T cells following ligation with CD86 induced IL-2 production whereas in T cell blasts there was no apparent induction of IL-2 production 6 or more days after the initial stimulation by PMA and Ionomycin. Although CD86 appeared to function in a similar way to CD80 there were differences in their efficiency. CD86 was more efficient in stimulating previously unactivated T cells (in conjunction with OKT3) to proliferate but was less efficient at increasing the proliferation of activated T cell blasts. It may be that each ligand has a specific role to play in the T cell immune response with CD86 being involved in the initial response and CD80 in augmenting the already activated T cells. The distribution of CD80 and CD86 to some extent confirms this suggestion as CD86 is found on unactivated cells but surface expression of CD80 occurs after activation.

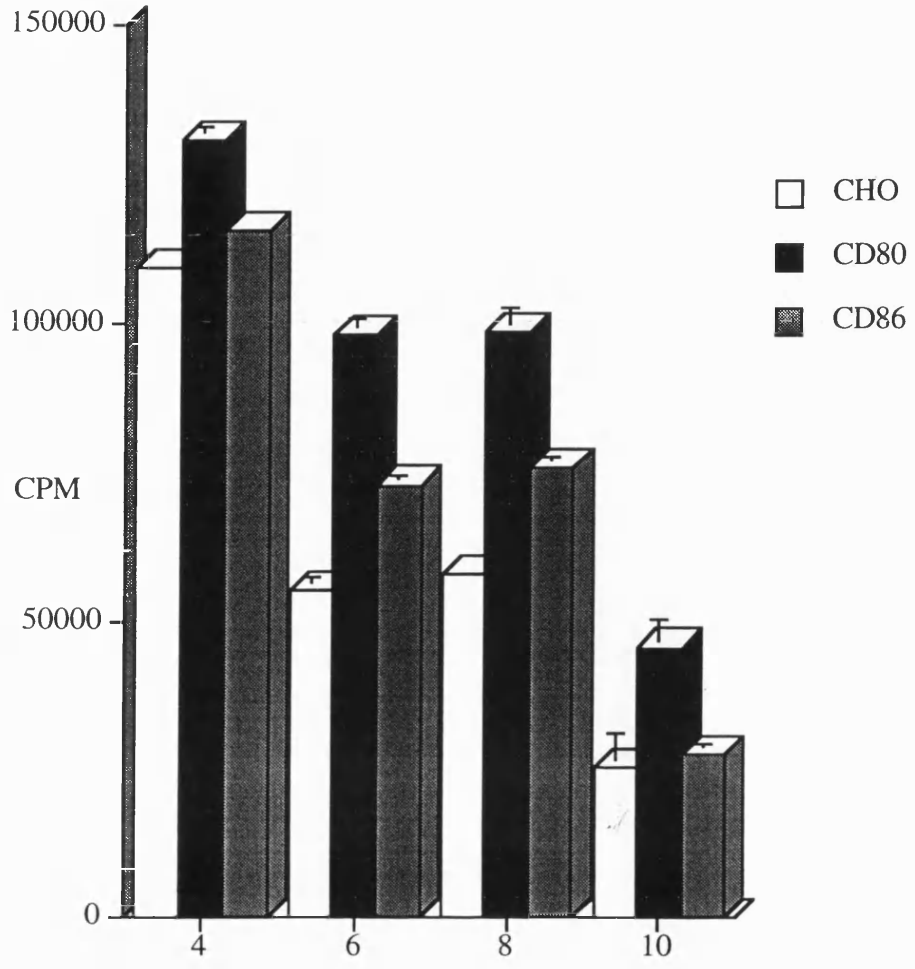


Figure 5.4 CD86 stimulation of T cell blasts over time.
2 x 10^4 PMA/Ionomycin T cell blasts at 4, 6, 8 and 10 days after their initial stimulation were cultured with 5 x 10^3 transfectants as shown. Proliferation was measured after 48 hours using ^3H thymidine incorporation . The data are from triplicate samples from a single representative experiment (n = 3). Additional data has been tabulated in appendix 5.

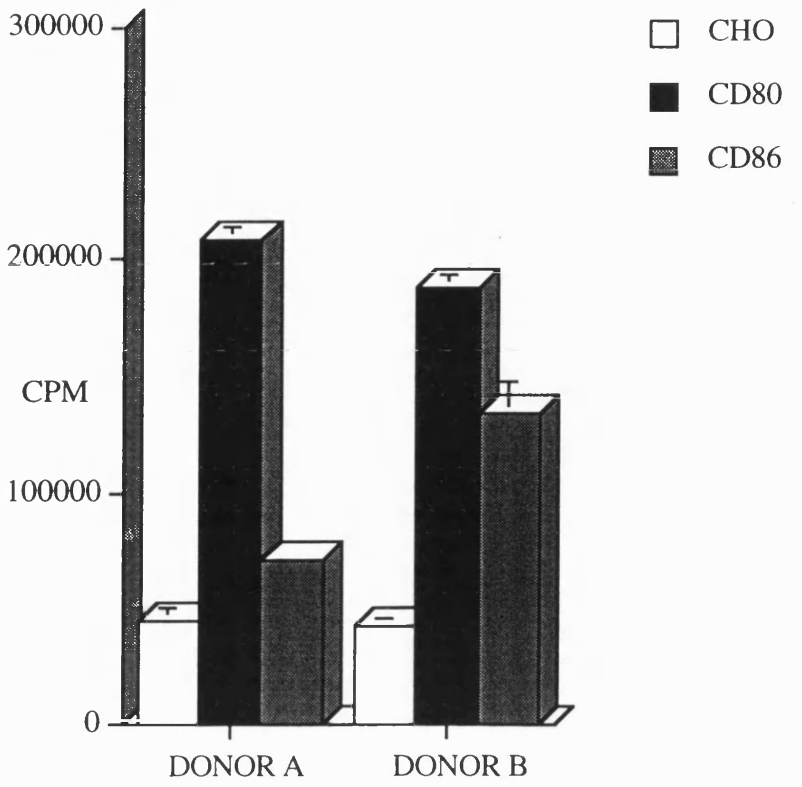


Figure 5.5 CD86 stimulation of T cell blasts.
5 x 10^4 T cell blasts (6 days post initial stimulation) from two different blood donors were cultured with 5 x 10^3 transfectants as shown. Proliferation was measured after 48 hours using ^3H thymidine incorporation.

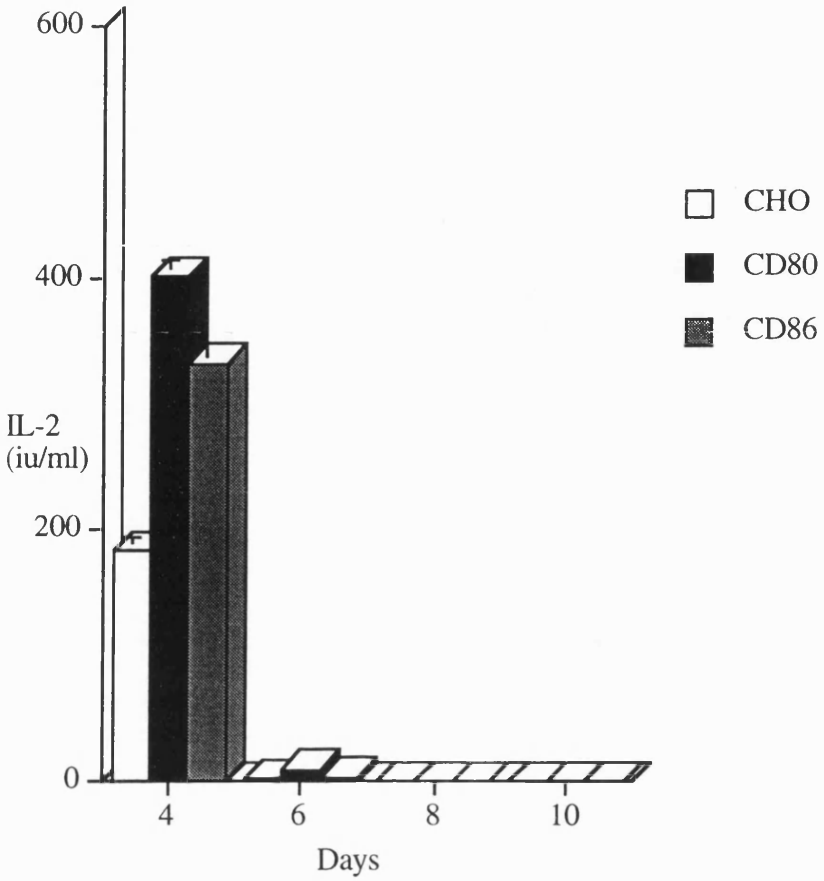


Figure 5.6 IL-2 production by T cell blasts in response to CD86 stimulation. 2 x 10^4 PMA/Ionomycin T cell Blasts at 4, 6, 8 and 10 days after their initial stimulation were cultured with 5 x 10^3 transfectants as shown. IL-2 production was measured after 48 hours using a CTLL bioassay. The data are from triplicate samples from a single representative experiment (n = 3).

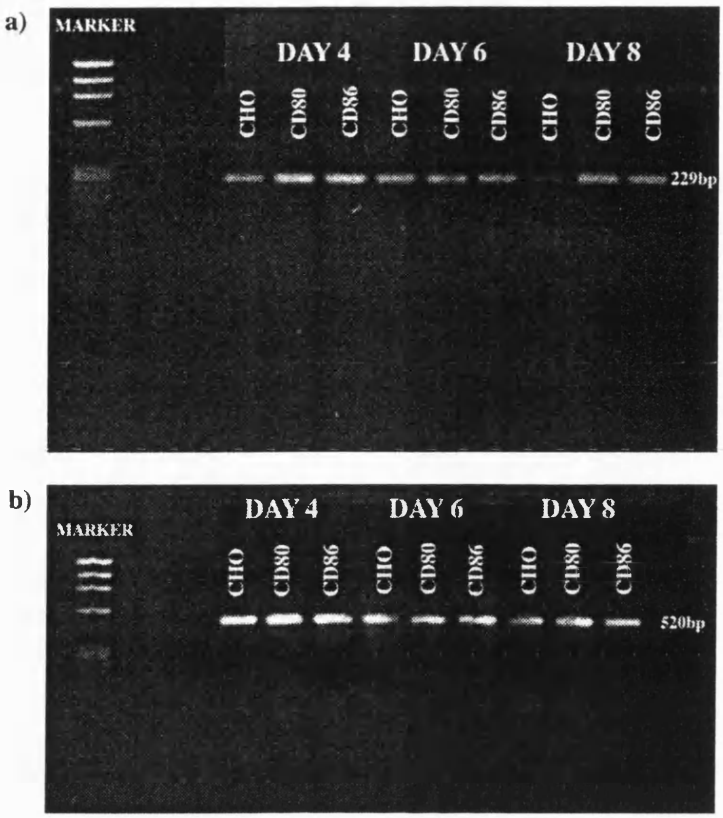


Figure 5.7 IL-2 transcription from T cell blasts stimulated by CD86 transfectants.

IL-2 transcription from T cell blasts stimulated by transfectants.

T cell blasts were produced (as detailed in figure 4.6) and 5 x 10^5 blasts (4, 6 or 8 days post stimulation) were cultured with 1 x 10^5 transfectants (CHO, CD80 or CD86) and the RNA extracted after 3 hours. The RNA was reverse transcribed and amplified by PCR using IL-2 primers. The resulting DNA was evaluated on a 2% agarose gel by electrophoresis. The marker ϕX 174 DNA digested with Hae lll was also run to indicate to size of the individual bands.

Figure 5.7a shows IL-2 transcription

Figure 5.7b shows GAPDH transcription

The research on mouse T cells discussed earlier suggests that CD86 may be the main provider of costimulation of T cells in conjunction with TCR signalling (Hathcock et al., 1994). This has also been seen in human T cells where anti-CD86 blocking antibodies were more effective at inhibiting a mixed leukocyte reaction (MLR) than anti-CD80 mAbs (Azuma et al., 1993b). Examination of the role of CD80 and CD86 in generating cytokine production has shown that CD86 costimulation preferentially induces IL-4 production particularly in naive T cells (Freeman et al., 1995). In repeatedly costimulated T cells both CD80 and CD86 induce both IL-2 and IL-4 but CD80 is the more potent stimulator of IL-2 (Freeman et al., 1995). The induction of IL-2 by repeatedly activating T cells seems to contradict the lack of IL-2 production by T cells blasts stimulated by CD80 and CD86 transfectants as demonstrated above and in Chapter 4. However it should be noted that the restimulation of the T cells by Freeman et al. was by MLR which would involve stimulation via both the TCR and CD28. This double signal is required for IL-2 production. More recent in vivo studies using double knock-out mice (i.e. lacking both CD80 and CD86 genes) has found that both CD80 and CD86 play a critical role in humoral immune responses particularly in immunoglobulin class switching (Borriello et al., 1997). Although this research is investigating the role CD28 plays in T cell responses following ligation by either CD80 and CD86, the knockout mice data clearly demonstrates that CD28 and its ligands also play a major role in B- cell generated immune responses.

The data generated so far have shown that CD28 is as essential molecule whose stimulation by CD80 and CD86 results in the generation and sustaining of T cell responses. The fact that there are two counter-receptors for CD28 which appear to generate similar signals resulting in T cell proliferation in resting T cells as well as augmenting T cell blast proliferation may suggest that one of these receptors is functionally redundant. However there are a number of differences between CD80 and CD86 which suggest they may perform different functions.

Distribution of CD80 and CD86 is markedly different, with CD80 expression being restricted to activated cells whereas CD86 is found on resting monocytes. Studies examining CD80 and CD86 expression on activated B cells shows marked differences in both the kinetics of expression and the induction of CD80 and CD86 expression which appears to depend on the stimulus applied (Hathcock et al., 1994). This difference in expression of

CD80 and CD86 suggested that perhaps CD86 was required for the initiation of an immune response and CD80 was required for amplification of the immune response. This is to some extent seen in this research where CD86 is more effective at providing costimulation to resting T cells than CD80 i.e. more effective in the initiation of T cell activation. However when already activated T cells are examined CD80 is more effective at augmenting T cell proliferation.

The functional outcome of CD80 and CD86 ligation in terms of cytokine production has also shown marked differences. Although both ligands are able to stimulate T cells to produce IL-2 and IFN-γ, CD86 preferentially stimulates IL-4 production (Freeman et al., 1995). As IL-4 is associated with the Th2 subpopulation of T helper cells, it may be that stimulation of T cells by CD86 results in the generation of $Th2$ T cells whereas CD80 stimulation results in $Th1$ cells being generated. This has been to some extent supported by research into the mouse model of experimental allergic encephalomyelitis (EAE). If CD80 interactions were blocked using mAbs the mice generated $Th2$ cells whereas if CD86 interactions were blocked $Th1$ cells were generated (Kuchroo et al., 1995).

It is well established that CD80 and CD86 can act as counter-receptors for CD28 resulting in the generation of signals which can induce IL-2 production and proliferation in resting T cells as well as sustaining proliferation of already activated T cells. However there is a CD28 homologue, CTLA-4 , which is not considered to be a redundant molecule. Evidence for CTLA-4 involvement in T cell activation has been predominantly from mouse studies where CTLA-4 signalling has been shown to act as a negative regulator of T cell activation. This negative regulation has been shown to be due to inhibition of IL-2 production and inhibiting proliferation by disruption of the cell cycle rather than induction of apoptosis (Krummel and Allison, 1996; Walunas et al., 1996). It is possible that a feature of CD80 and CD86 interactions with T cells could be that CD86 is predominantly involved with CD28 whereas CD80 is the main counter-receptor for CTLA-4. This is seen to some extent by the binding kinetics of CD80 and CD86 to CTLA-4. Both CD80 and CD86 bind CTLA-4 with similar affinity but the rate of dissociation of CD80 from CTLA-4 is much slower than CD86 (Linsley et al., 1994). The surface expression of CTLA-4 and CD80 is also similar - both are expressed following T cell activation i.e. both receptor and counter-receptor are only present following activation. The involvement of CTLA-4 in T cell activation is still

being investigated and the interactions between of CD80, CD86 and their receptors CD28 and CTLA-4 may prove to be highly complex. It is likely that CD28 and CTLA-4 as well as CD80 and CD86 have distinct roles to play in the control of T cell activation.

Chapter 6

CD28 Signalling

The ability of CD28 to provide the correct signals to the T cell requires a sophisticated signalling system. Although the signalling mechanism of CD28 was not examined directly, it was possible using a selective inhibitor to determine the effect on T cell function of disrupting CD28 signalling. A major insight into CD28 signalling was the observation that ligation of CD28 by CD80 led to an increase in phosphatidylinositol-(3,4,5)-trisphosphate production (Ward et al., 1993). This suggested that the CD28 signalling pathway utilised phosphoinositide 3 kinase (PI3-kinase). Further research demonstrated that ligation of CD28 by CD80 resulted in the p85 subunit of PI3-kinase associating with CD28 possibly via the YXXM motif in the cytoplasmic tail of CD28 (Ward et al., 1995). The association of PI3-kinase with CD28 signalling meant it was possible to utilise inhibitors of PI3-kinase such as wortmannin to examine the effect of disruption of this CD28 signalling pathway on T cell activity.

Effect of wortmannin on CD28 costimulation of purified T cells.

Wortmannin (a fungal metabolite) is known to bind irreversibly to the p110 catalytic subunit of PI3-kinase and inhibit the catalytic activity of the enzyme (Arcaro and Wymann 1993). To test whether disruption of PI3-kinase activity had any effect on T cell activation, wortmannin was added to T cells cultured in the presence of OKT3 10 minutes prior to the addition of costimulatory transfectants. This allowed the wortmannin time to bind to the p110 subunit of PI3-kinase prior to costimulation of the T cells. As T cell costimulation has been shown to be CD28 dependent, this is a reasonable test of whether PI3-kinase might be involved in CD28 signalling.

The experiment revealed that wortmannin, at concentrations as low as 1nM, could inhibit T cell activation, in response to OKT3 and CD80 costimulation, resulting in reduced proliferation (Figure 6.1). The reduction in proliferation was irreversible as measuring proliferation on a daily basis following incubation of T cells with wortmannin (1μM) in the presence of OKT3 and CD80 transfectants showed no diminishing of the inhibitory effects of wortmannin (Figure 6.2).

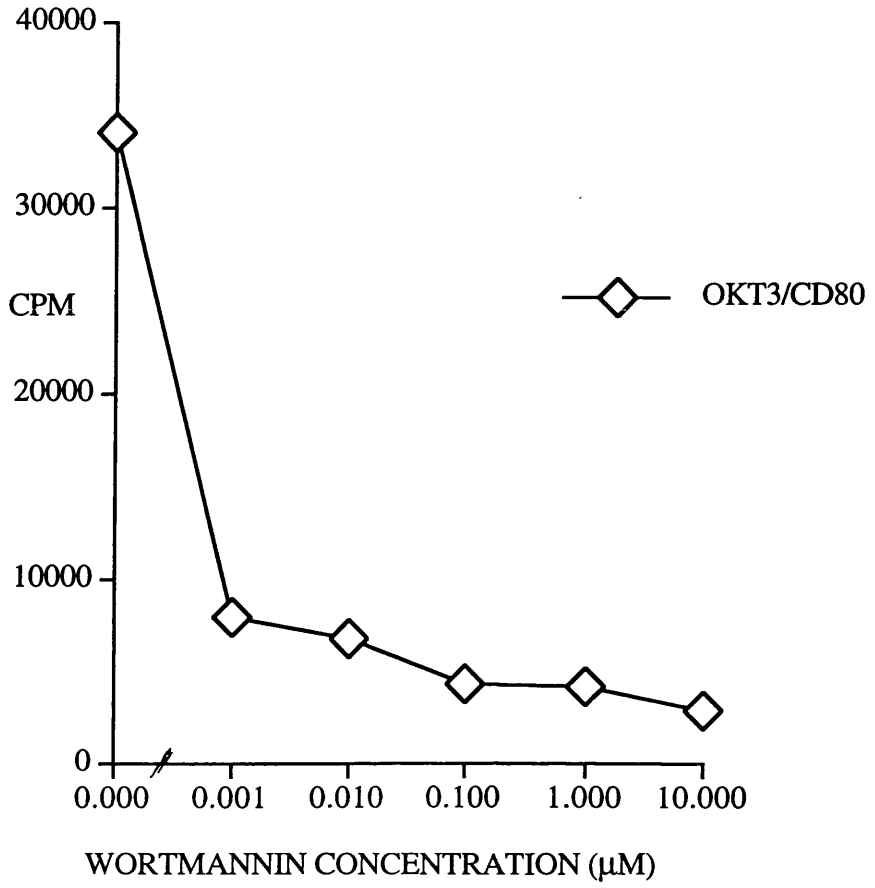


Figure 6.1: Wortmannin inhibition of T cell proliferation
Purified T cells (5 x 10^4) were cultured with CD80 transfectants (2 x 10^4) in the presence of OKT3 (1μg/ml) and wortmannin at various concentrations Proliferation was measured after 72 hours using ^3H thymidine incorporation. The data are from triplicate samples from a single representative experiment (n = 4).

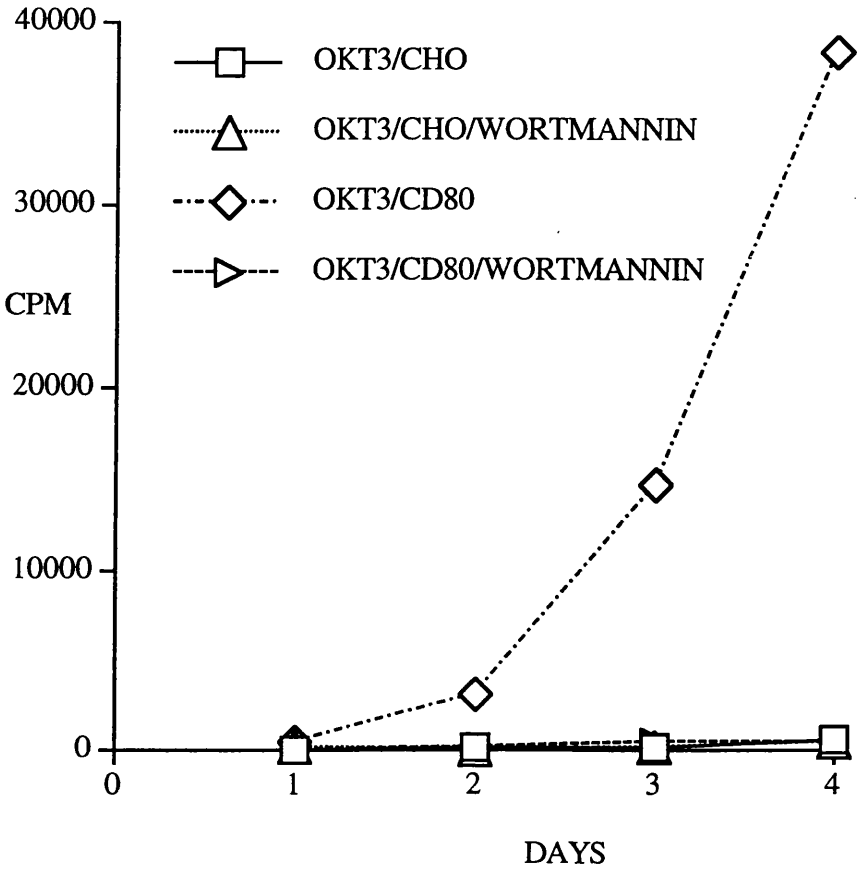


Figure 6.2: Kinetics of wortmannin inhibition of T cell proliferation .
Purified T cells (2 x 10^4) were cultured with transfectants (2 x 10^4), either CHO or CD80 in the presence of OKT3 (1μg/ml) and the presence / absence of wortmannin (1μM). Proliferation was measured every 24 hours using ^3H thymidine incorporation .The data are from triplicate samples from a single representative experiment (n = 3).

To test whether the efficiency of blocking PI3-kinase activity was affected by preparing wortmannin in advance, the drug solution was prepared and incubated overnight at 37°C before being added to a T cell stimulation experiment as before. The wortmannin which had been pre-incubated for 24 hours was unable to prevent T cell activation in the presence of OKT3 and CD80 transfectants (Figure 6.3b). However freshly prepared wortmannin was able to inhibit T cell proliferation (Figure 6.3a). This clearly demonstrated that the ability of wortmannin to block T cell activation could be impaired if the PI3-kinase inhibitor was prepared in advance. In all experiments wortmannin was freshly prepared and used immediately to overcome the problem of its instability in aqueous solutions.

Although wortmannin was shown to inhibit T cell proliferation it was possible that wortmannin was not blocking CD28 signalling but was preventing proliferation by disrupting the IL-2 receptor signalling pathway as T cells proliferate in response to IL-2 ligation of the IL-2R. To determine whether wortmannin was blocking CD28 signalling or IL-2R signalling two experiments were carried out. Firstly the effect of wortmannin on IL-2 production by T cells stimulated with OKT3 and CD80 was examined. As a major function of CD28 signalling is to increase IL-2 mRNA transcription and increase IL-2 mRNA stability, loss of IL-2 production would indicate that the CD28 signalling pathway was being blocked by wortmannin. To determine if wortmannin was blocking IL-2R signalling rather than CD28 signalling, addition of the PI3-kinase inhibitor should prevent the IL-2 stimulated proliferation of T cell blasts.

The results of the experiments clearly showed that wortmannin blocked IL-2 production in T cells stimulated with OKT3 and CD80 (Figure 6.4b) but had no effect on the stimulation of T cell blasts using exogenous IL-2 (Figure 6.4a). This suggests that wortmannin was blocking the signalling pathway which led to activation of the IL-2 gene and not the downstream event of IL-2 receptor signalling. The lack of inhibition of IL-2 specific proliferation of T cell blasts by wortmannin also demonstrated that its effect on purified T cells was not due to non-specific toxicity.

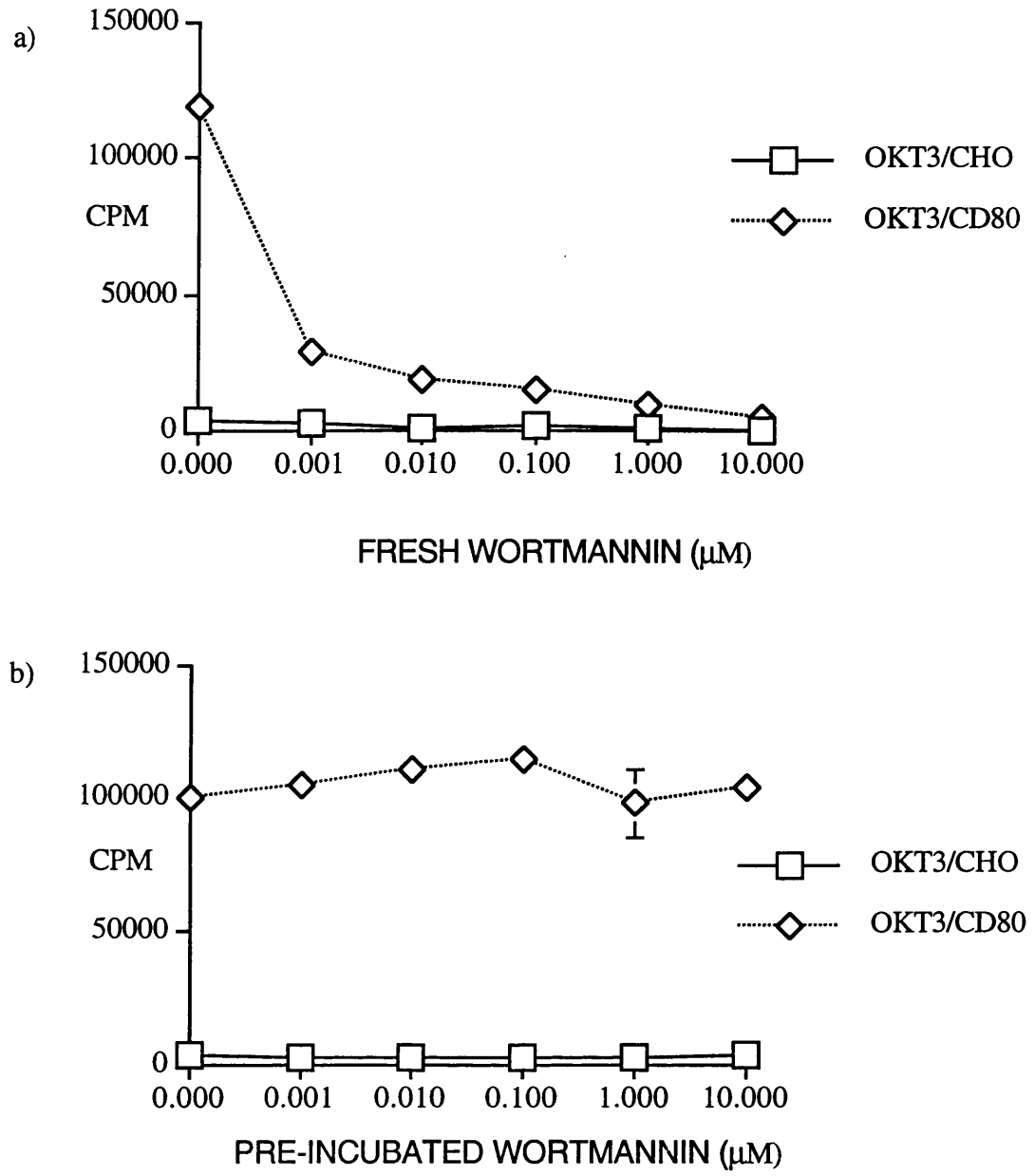


Figure 6.3: Stability of wortmannin in aqueous solution.
Purified T cells (5 x 10^4) were cultured in the presence of OKT3 (1μg/ml) with either CHO or CD80 transfectants (2 x 10^4) with either freshly prepared wortmannin or wortmannin which had been pre-incubated at 37°C for 24 hours prior to its use. Proliferation was measured using ^3H thymidine incorporation after 48 hours. The data are from triplicate samples from a single representative experiment (n = 3).

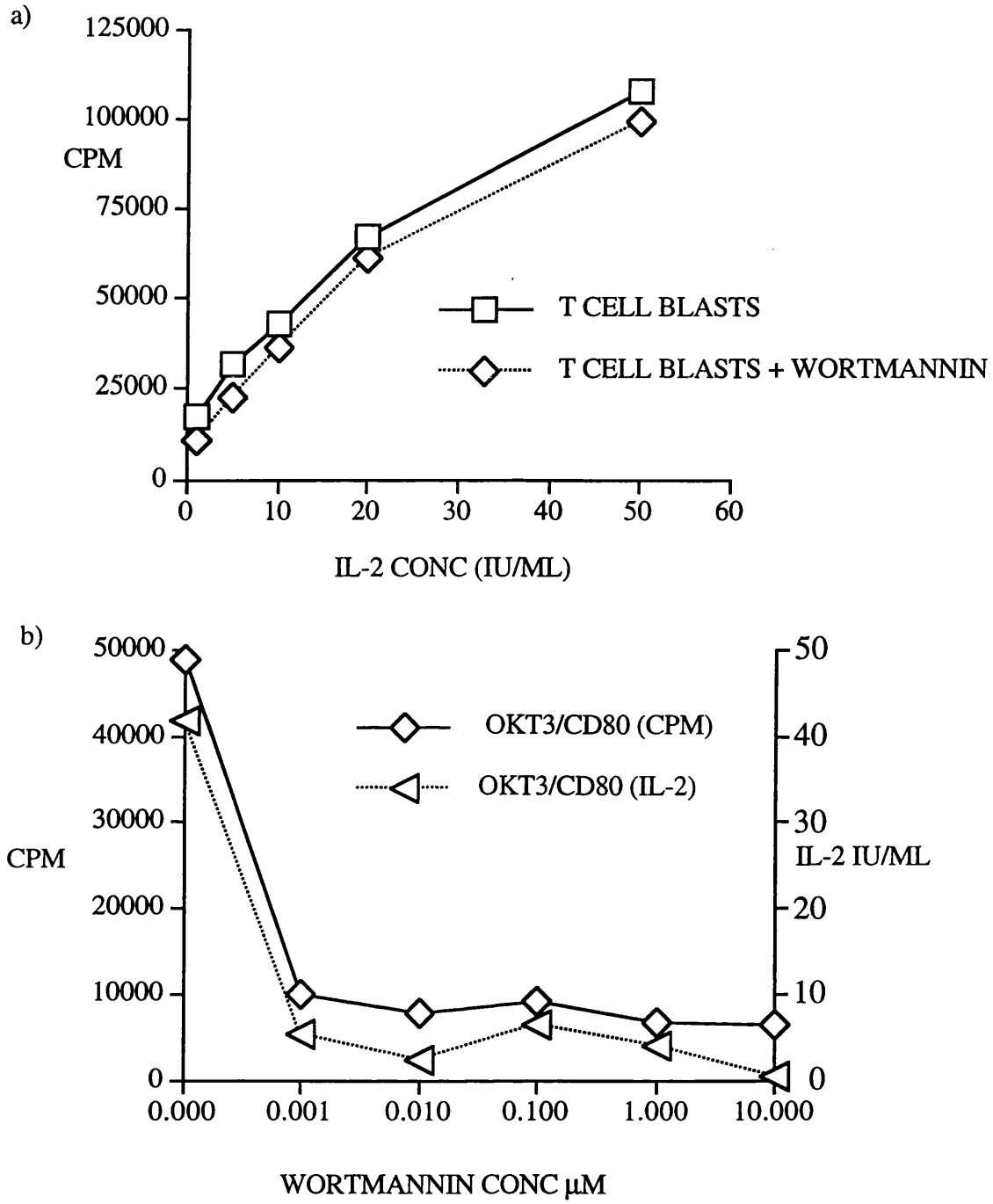


Figure 6.4 : Wortmannin inhibition of T cell signalling.
a) T cell blasts (5 x 10^4) were induced to proliferate in response to increasing amounts of IL-2 in the presence/absence of wortmannin (10µM). Proliferation was measured using ^3H thymidine incorporation after 48 hours in culture.
b) Purified T cells (5 x 10^4) were cultured with CD80 transfectants (2 x 10^4) and OKT3 (1µg/ml) in the presence of increasing concentrations of wortmannin. Proliferation and IL-2 production were measured using ^3H thymidine incorporation and a CTLL bioassay respectively after 48 hours The data are from triplicate samples from a single representative experiment (n = 3).

Although the signalling pathways utilised by CD28 were not examined directly, it was possible to show that wortmannin could functionally effect the stimulation of previously unactivated T cells and that this involved inhibition of IL-2 production. This inhibition indicated that the blockade occurred during the initial signals required for IL-2 production and not a later signal generated when IL-2 bound to its receptor.

Subsequent research by Ueda et al., (1995) has also shown that wortmannin was able to block IL-2 production in primary T cells when stimulated by anti-CD3 mAbs and either anti-CD28 mAbs or CD80 transfectants confirming the results shown in Figure 6.4b. However, the T cells used by Ueda et al., were rested T cell blasts (which had been generated using anti-CD3 and anti-CD28 mAbs) and subsequently restimulated. The sensitivity to wortmannin of these rested T cell blasts contradicts the results found when wortmannin was used to block IL-2 stimulation of T cell blasts (Figure 6.4a). The difference in the sensitivity of T cell blasts to wortmannin may depend on whether they are activated or resting i.e. may be due to the differences in the cell cycling of rested and activated T cell blasts. The T cell blasts which were being IL- 2 stimulated were already dividing in the absence of IL-2 whereas the rested T cell blasts were not actively cell cycling and on stimulation via CD3 and CD28 the T cell blasts would then enter the cell cycle. This induction to cell cycling may involve signalling via PI3-kinase which could then be blocked by the addition of wortmannin. In the case of the activated T cell blasts which were already cell cycling, it is possible that the signalling pathways used in these cells do not require PI3-kinase and therefore T cell blasts are insensitive to wortmannin.

This difference in response to wortmannin depending on the activation state of the T cell is also seen when wortmannin is used to block CD28 signalling in Jurkat cells. Wortmannin has been shown to have no effect on IL-2 secretion in Jurkat cells following ligation of CD28 in the presence of PMA and Ionomycin (Lu et al., 1995). As Jurkat cells are an activated cell line and therefore actively dividing their insensitivity to wortmannin could also be due to the signalling pathways used in the cell cycle not requiring PI3-kinase.

The functional studies undertaken to examine the effect of wortmannin on T cell activation have shown that not only was T cell proliferation inhibited but this was achieved by blocking IL-2 production. As wortmannin is known to bind irreversibly to the p110 subunit of PI3- kinase and inhibit its activity this

would suggest that PI3 - kinase is involved in T cell signalling. Additionally, as CD28 signalling is involved in the induction of IL-2 gene transcription (by increasing transcription and the stability of the mRNA as well as induction of gene transcription factors such as the CD28RC) this would suggest that PI3-kinase may be associated with the CD28 signalling pathway.

There have been a number of biochemical studies showing the relationship between CD28 and PI3-kinase. Ligation of CD28 by CD80 resulted in the formation of D - 3 phosphoinositides (which are the products of PI3-kinase activity) in the absence of TCR/CD3 activation (Ward et al., 1993). Subsequently, it was demonstrated that CD28 was directly associated with the p85 subunit of PI3-kinase and that this association was ligand dependant. (Ward et al., 1995). The lipid kinase activity associated with CD28 following ligation by CD80 was shown directly using HPLC analysis to be due to PI3-kinase as well as indirectly by increased formation of D-3 phosphoinositides. The PI3-kinase activity was abrogated by the addition of 100nM wortmannin but the physical association of CD28 and the regulatory p85 subunit was unaffected (Ward et al., 1995). There is further evidence of PI-3 kinase association with CD28 in two studies examining CD28 signalling in Jurkat cells. An examination of the protein tyrosine kinases (PTKs) involved in phosphorylating the YMNM motif in the cytoplasmic tail of CD28 demonstrated that $p56^{lck}$ and $p59^{fyn}$ were capable of phosphorylating the YMNM motif which led to recruitment of the p85 subunit of PI3-kinase (Raab et al., 1995). It has also been shown that the association between CD28 and PI3-kinase in Jurkat cells can be inhibited by the phorbol ester PMA. This inhibition was found to persist and blocked PI3-kinase activity following stimulation of the cells with anti-CD28 mAbs (Hutchcroft et al., 1995). The same study also demonstrated the lack of inhibition of wortmannin on IL-2 induction following stimulation of Jurkat cells with PMA and anti-CD28 mAbs. As PMA activates protein kinase C and wortmannin was unable to block IL-2 production by Jurkat cells this suggests that PI3-kinase is not involved in CD28 dependent IL-2 production in Jurkat cells. The conclusion that CD28 signalling in Jurkat cells may not involve PI3-kinase suggests that there may be more than one signalling pathway following CD28 ligation depending on the state of activation of the T cells. This has also been shown by the lack of effect of wortmannin on IL-2 stimulation of T cell blasts (Figure 6.4a). It was therefore important to examine the effect of wortmannin on CD28 only stimulation of T cell blasts. This would give an insight into whether PI3-kinase was involved in CD28 signalling in T cell blasts in the

same way as it is involved in CD28 signalling in previously unstimulated T cells.

Effect of wortmannin on stimulation of T cell blasts.

Having previously demonstrated that T cell blasts could be stimulated by CD80 transfectants resulting in increased proliferation, it was now possible to investigate CD28 signalling and determine whether PI3-kinase was involved in the CD28 signalling pathway in activated T cell blasts.

T cell blasts were cultured in the presence or absence of wortmannin in conjunction with transfectants. The results in Figure 6.5 show that even at a concentration as high as 10μM, wortmannin had no effect on the augmentation of T cell blast proliferation by CD80 costimulation. This inability of wortmannin to block CD28 signalling was irrespective of the time after initial mitogenic stimulation that costimulation was administered (Figure 6.6). Similarly wortmannin had no obvious effect on IL-2 production by the T cell blasts with high levels of IL-2 being produced by T cell blasts 4 days post initial stimulation and negligible IL-2 by older T cell blasts. (Figure 6.7). These data suggest that PI-3 kinase was not involved in the CD28 signalling pathway in T cell blasts.

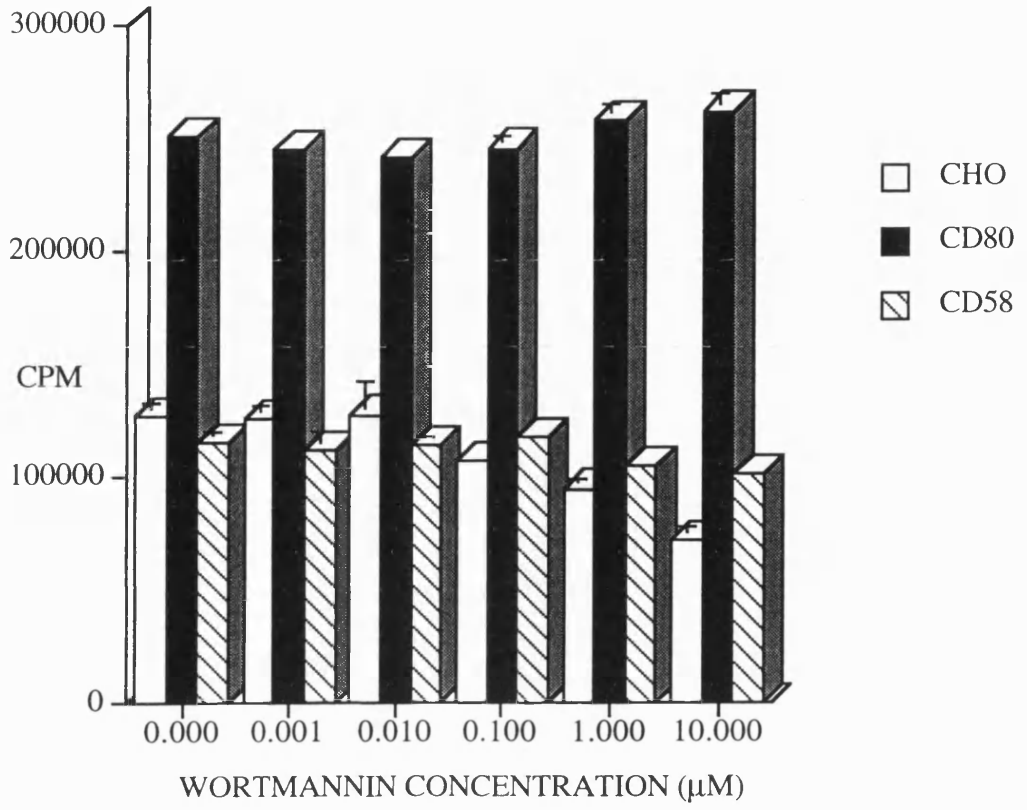


Figure 6.5 Wortmannin inhibition of T cell blasts.
5 x 10^4 PMA/Ionomycin T cell blasts 7 days post initial stimulation were cultured with 2 x 10^4 transfectants in the presence of increasing concentrations of wortmannin. Proliferation was measured using ^3H thymidine incorporation after 48 hours. The data are from triplicate samples from a single representative experiment (n = 5).

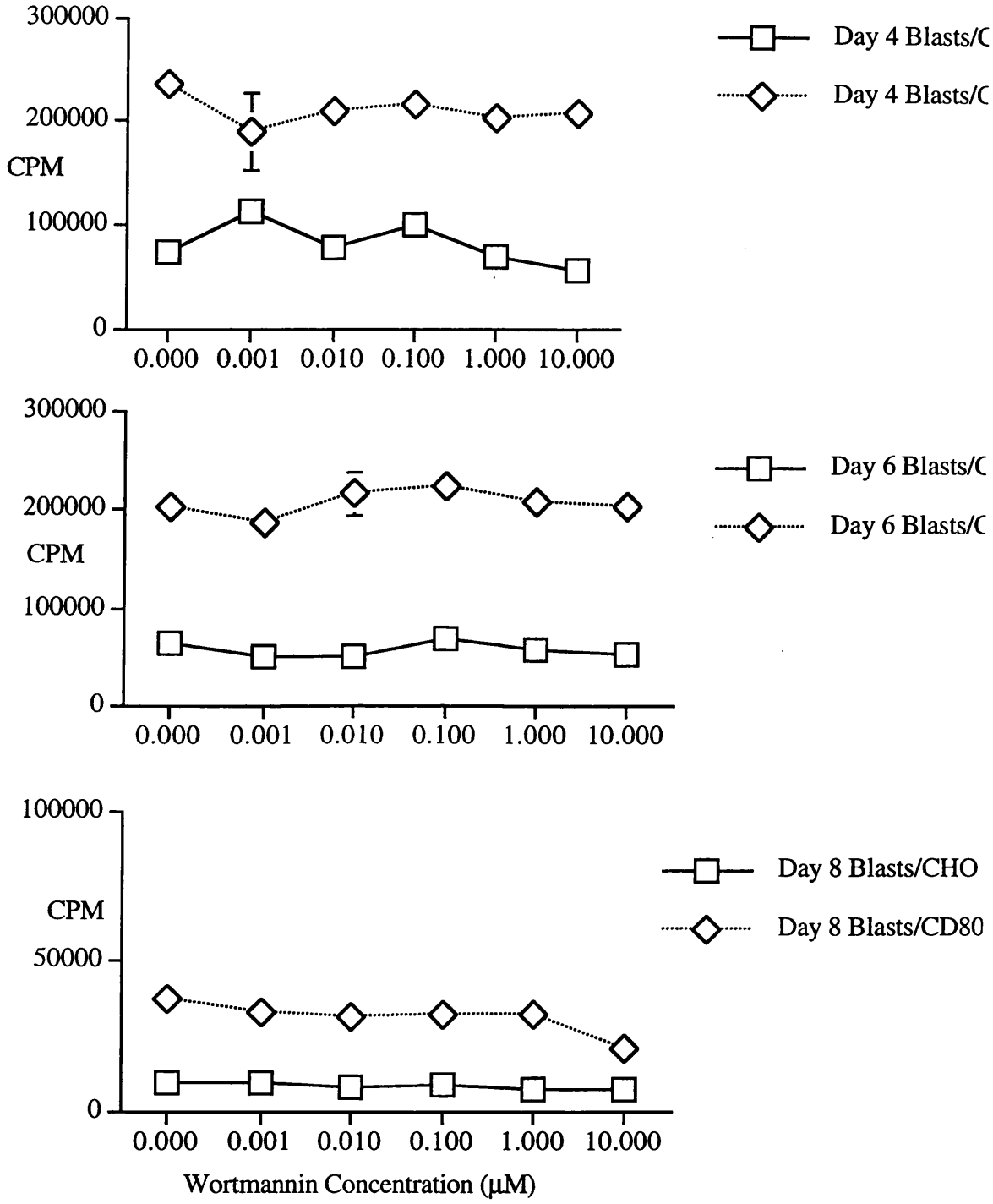


Figure 6.6 Wortmannin inhibition of T cell blasts over time.
5 x10^4 T cell blasts (4, 6 and 8 days post initial stimulation) were cultured with 1 x 10^4 transfectants in the presence of increasing concentrations of wortmannin. Proliferation was measured using ^{3}H thymidine incorporation after 48 hours. The data are from triplicate samples from a single representative experiment (n = 3).

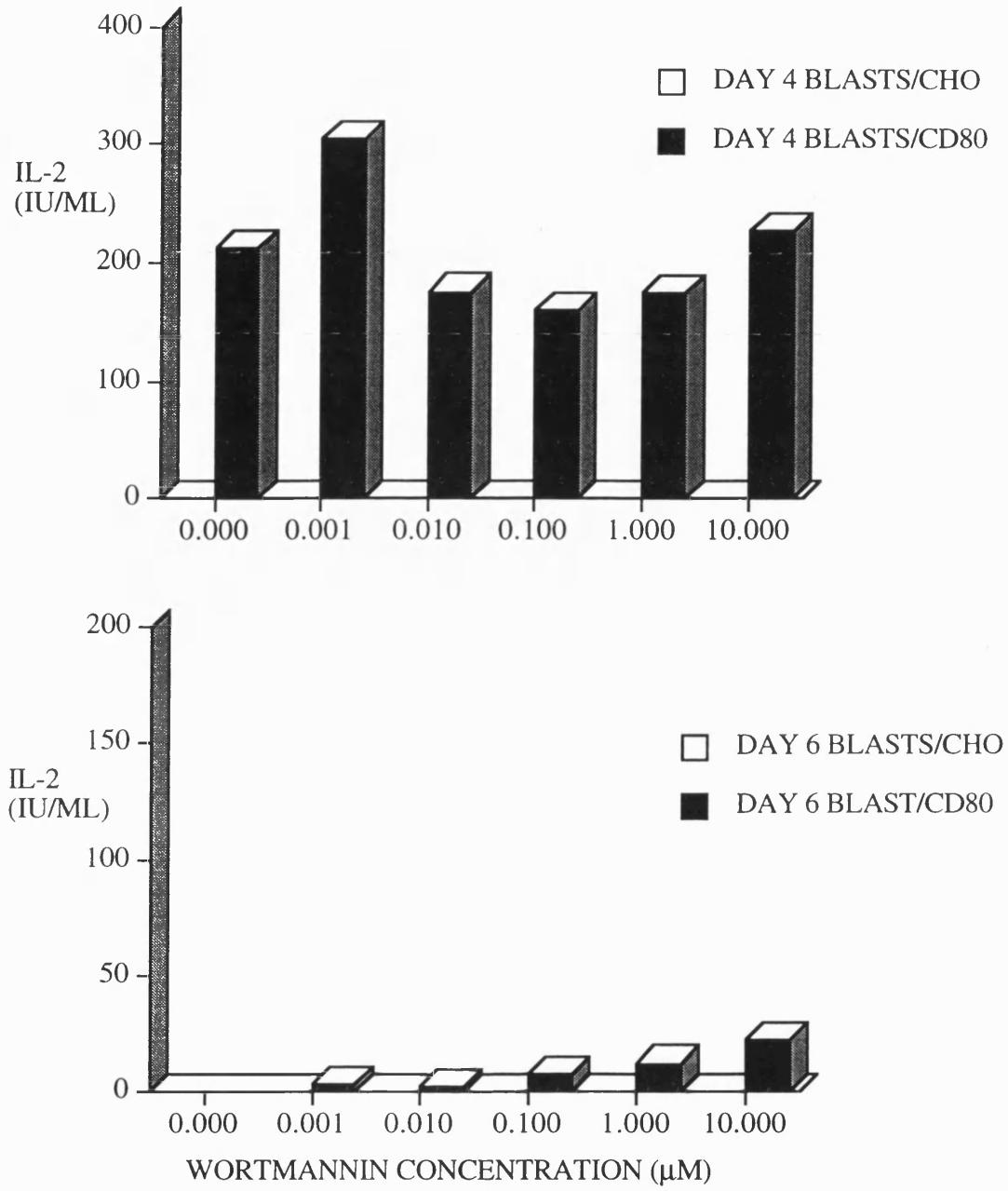


Figure 6.7 Wortmannin inhibition of IL -2 production by T cell Blasts.
IL-2 production by T cell blasts (5 x 10^4), cultured with transfectants (1 x 10^4) and in the presence of increasing concentrations of wortmannin, was measured after 48 hours using a CTLL bioassay (see Figure 6.6 for proliferation data). The data are from triplicate samples from a single representative experiment (n = 3).

The lack of inhibition of wortmannin on CD28 stimulation of T cell blasts is in line with research on Jurkat and other activated T cells. The slight induction of IL-2 production shown in Day 6 T cell blasts at high concentrations of wortmannin (Figure 6.7) coincides with results obtained when Jurkat cells were cultured with wortmannin where a small induction of IL-2 secretion was seen (Ueda et al., 1995). The lack of inhibition of wortmannin on enhancement of T cell proliferation as a result of CD28 ligation suggests that PI3-kinase was not involved in the CD28 signalling pathway in activated T cells. In chapter 5, wortmannin has already been shown to inhibit IL-2 production in previously unstimulated T cells (cultured with OKT3 and CD80 transfectants) which suggests that the IL-2 induction pathway in those T cells involves PI3-kinase. Lack of IL-2 production by T cell blasts (Day 6 or older - Figure 4.4) would suggest that the IL-2 production signalling pathway was not involved in the CD28 signals generated in the activated T cell blasts and therefore no PI3-kinase activity was occurring. However the ability of activated T cell blasts to respond to CD28 signalling in the absence of IL-2 suggests that an alternative signalling pathway is involved and lack of inhibition by wortmannin suggests that this alternative signalling pathway generated by CD28 does not involve PI3-kinase.

Recent research has shown that CD28 can utilise a sphingomyelinase signalling pathway (Edmead et al., 1996). The research examined the induction of transcription factors and the sensitivity of this induction to enzyme inhibitors. CD28 ligation was shown to generate the AP-1 transcription factor and since this AP-1 induction was wortmannin sensitive, PI3-kinase was probably involved in the signalling pathway. However induction of the transcription factor, NF-kB, by CD28 was insensitive to wortmannin but could be inhibited by chloroquine (which modifies lysosomal pH and can inhibit acidic sphingomyelinase) suggesting that PI-3 kinase is not involved in this signalling pathway - acidic sphingomyelinase was used instead. IL-2 production requires the presence of a number of transcription factors including AP-1, NF-kB and NF -AT. Using T cell blasts and stimulating them with CD80 transfectants resulted in the production of AP-1 and NF-kB only and no IL-2 production. This suggests that TCR engagement and signalling is required for NF-AT production and all three transcription factors required for IL-2 production. Using TCR only or TCR and CD28 stimulation in T cell blasts 6 days or more post initial stimulation all three transcription factors were induced and IL-2 production could be detected which confirmed the necessity of NF-AT for IL-2 production.

The ability of CD28 to use a different signalling pathway - acidic sphingomyelinase could perhaps explain the lack of effect of wortmannin on T cell blasts stimulated by CD80 transfectants (Figure 6.5). It may be that mitogen stimulation of PBMCs using PMA and Ionomycin results in the generation of the transcription factors AP-1, NF-kB and NF-AT and therefore IL-2 production (as detected by the bioassay for Day 4 blasts). The T cells then proliferate in response to IL -2. With time the level of proliferation of the T cell blasts declines due to a decrease in the level of IL-2 production (as seen by lack of detection of IL-2 by the bioassay in older T cell blasts). This decline in IL-2 production in the T cell blasts is similar to the profile of IL-2 production by purified T cells stimulated with OKT3 and CD80 transfectants (Figure 3.8b). As already discussed in Chapter 3 this may be due to the IL-2 gene being switched off as the original stimulation signals become quiescent. Alternatively, the upregulation of CTLA-4 on T cell activation (as well as CD80 and CD86) may result in T cell - T cell interactions and the generation of negative signals by CTLA-4 which inhibit production of IL-2.

However, when the T cell blasts were stimulated through CD28 using CD80 transfectants, proliferation of older T cell blasts (Day 6 and Day 8) was augmented in the absence of increased IL-2 production. It is possible that CD28 signalling utilised the sphingomyelinase pathway resulting in production of the AP-1 and NF- kB transcription factors. Lack of the transcription factor NF-AT due to lack of TCR stimulation would result in no induction of IL-2 gene transcription as all three transcription factors are required. The T cell blasts may be proliferating in response to another cytokine. It has been demonstrated elsewhere (Freeman et al., 1995) that CD86 and to a lesser extent CD80 were able to induce production of IL-4 in repeatedly stimulation T cells. In that research the T cell blasts were able to generate IL-2 due to restimulation involving TCR signalling and therefore the generation of NF-AT. However as one of the transcription factors associated with the IL-4 gene promoter is NF-AT it is unlikely that IL-4 is involved in augmenting older T cell proliferation by stimulation through CD28 alone. A method of determining whether cytokines were involved in augmenting proliferation of T cell blasts in response to ligation of CD28 would be supernatant transfer experiments. If the augmented T cell blasts proliferation, in response to CD28 stimulation, was cytokine driven then the supernatant from these T cells should contain unbound cytokine which if added to T cell blasts (but not stimulated by C80 or CD86 transfectants) should result in augmented proliferation.

The requirement for TCR signalling to generate the transcription factor NF-AT (which is essential for IL-2 production) may also help explain the inhibitory effect of wortmannin on restimulated rested T cell blasts as demonstrated by Ueda et al., (1995). As the rested T cell blasts were restimulated via the TCR and CD28 it is likely that all three transcription factors were generated. The inhibition of PI3-kinase by the addition of wortmannin may have inhibited induction of NF-AT and so prevented IL-2 production. As NF-AT induction requires TCR engagement and signalling , it is possible that the inhibitory effect of wortmannin was due to inhibition of PI3-kinase activity known to be associated with the TCR (Ward et al., 1992). This could also be true for wortmannin inhibition of proliferation of unstimulated T cells stimulated by OKT3 and CD80 (Figure 6.4b). The lack of IL-2 production may be due to inhibiting NF-AT transcription caused by blocking the PI3-kinase activity associated with TCR signalling and not with CD28 signalling. However as PI3-kinase has been shown to be associated with CD28 and the enzymatic activity of PI3-kinase generated following CD80 ligation of CD28 could be inhibited by nanomolar quantities of wortmannin (Ward et al., 1995) it is possible that wortmannin inhibition of proliferation of T cells (in response to OKT3 and CD80) is due to blocking PI3-kinase signals generated by both the TCR and CD28.

Effect of wortmannin on CD86 stimulation of T cells

It has already been demonstrated (Chapter 5) that either CD28 ligand is able to costimulate purified T cells to proliferate in conjunction with OKT3. Both ligands have also been shown to augment proliferation in T cell blasts following ligation with CD28. Comparison of the two ligands demonstrated that CD86 was more effective at costimulating purified T cells whereas CD80 was more effective at augmenting proliferation in T cell blasts. It was important to determine whether the effects of wortmannin on CD28 signalling in response to CD86 ligation were the same as that seen when CD80 was used to provide the stimulus to CD28. If there were any differences, this would give an indication that the signals generated by CD28 in response to CD86 were different to those generated in response to CD80 ligation.

When purified T cells were cultured with OKT3 and either CD80 or CD86 transfectants in the presence of wortmannin, inhibition of proliferation occurred (Figure 6.8). Although proliferation was inhibited regardless of

which ligand was present, the concentration of wortmannin required to inhibit proliferation due to CD86 costimulation was greater than for CD80. It has already been demonstrated that CD86 costimulation generated higher levels of proliferation than CD80. This would suggest that the signals generated by CD28 in response to CD86 were more potent than those following CD80 ligation. As a result a higher concentration of wortmannin would be required to inhibit the stronger signals generated in response to CD80 ligation. As has already been stated in the comparison of CD80 and CD86 stimulation of T cell blasts (Chapter 5), the stronger signals generated by CD28 in response to CD86 ligation were probably not due to increased affinity of CD28 for CD86 rather than CD80. The levels of receptor binding by the ligands is similar with CD86 binding levels 2 -3 fold less than CD80 (Linsley et al., 1994). However the binding kinetics data was determined using the chimeric molecules CD28-Ig and CTLA-4-Ig to bind to CD80 and CD86 CHO transfectants. It has been demonstrated that binding of C28 and also CTLA-4 with their ligands is in fact oligomeric (i.e. a disulphide homodimer of CD28 or CTLA-4 binds two CD80 or CD86 monomers) (Linsley, 1995), the affinity data obtained using these chimeric molecules may not represent the true affinities of CD80 and CD86 for CD28 and CTLA-4. Unfortunately, the differences in the strength of signal generated by CD28 following CD80 and CD86 ligation are not consistent (i.e. CD86 is better at providing costimulation to naive T cells whereas CD80 is better at stimulation of already activated T cells). It is likely that factors other than the affinity of CD80 and CD86 for their ligands may be involved.

As with CD80 stimulation of T cell blasts, wortmannin had no effect on CD86 stimulation of T cell blasts even at concentrations as high as 10μM (Figure 6.9). This would suggest that like CD80, CD86 ligation of CD28 does not involve signalling through PI-3 kinase. As has already been discussed above the signals generated by CD28 may involve a sphingomyelinase pathway and not PI3-kinase. The data obtained using CD86 ligation of CD28 on T cell blasts were no different to that obtained using CD80 transfectants - i.e. T cell blast proliferation was augmented in response to CD28 ligation and this proliferation was not inhibited by wortmannin. Although this does not confirm that CD80 and CD86 ligation of CD28 generates the same signals it does confirm that the signals generated do not involve PI3-kinase.

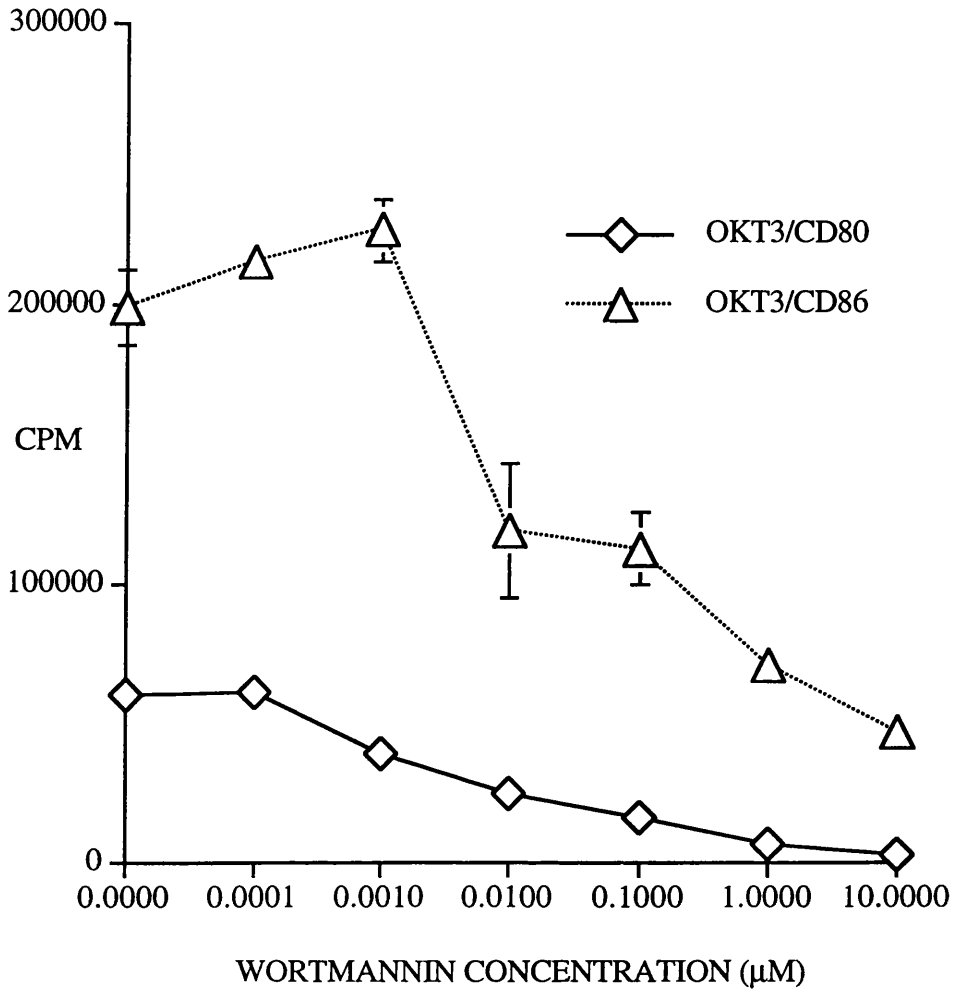


Figure 6.8 Inhibition of T cell proliferation by wortmannin.
5 x 10^4 T cells were cultured with OKT3 (1μg/ml) and 2 x 10^4 transfectants in the presence of increasing dosages of wortmannin as shown. Proliferation was measured after 48 hours by ^3H thymidine incorporation. The data are from triplicate samples from a single representative experiment (n = 3). Additional data are shown in Appendix 5.

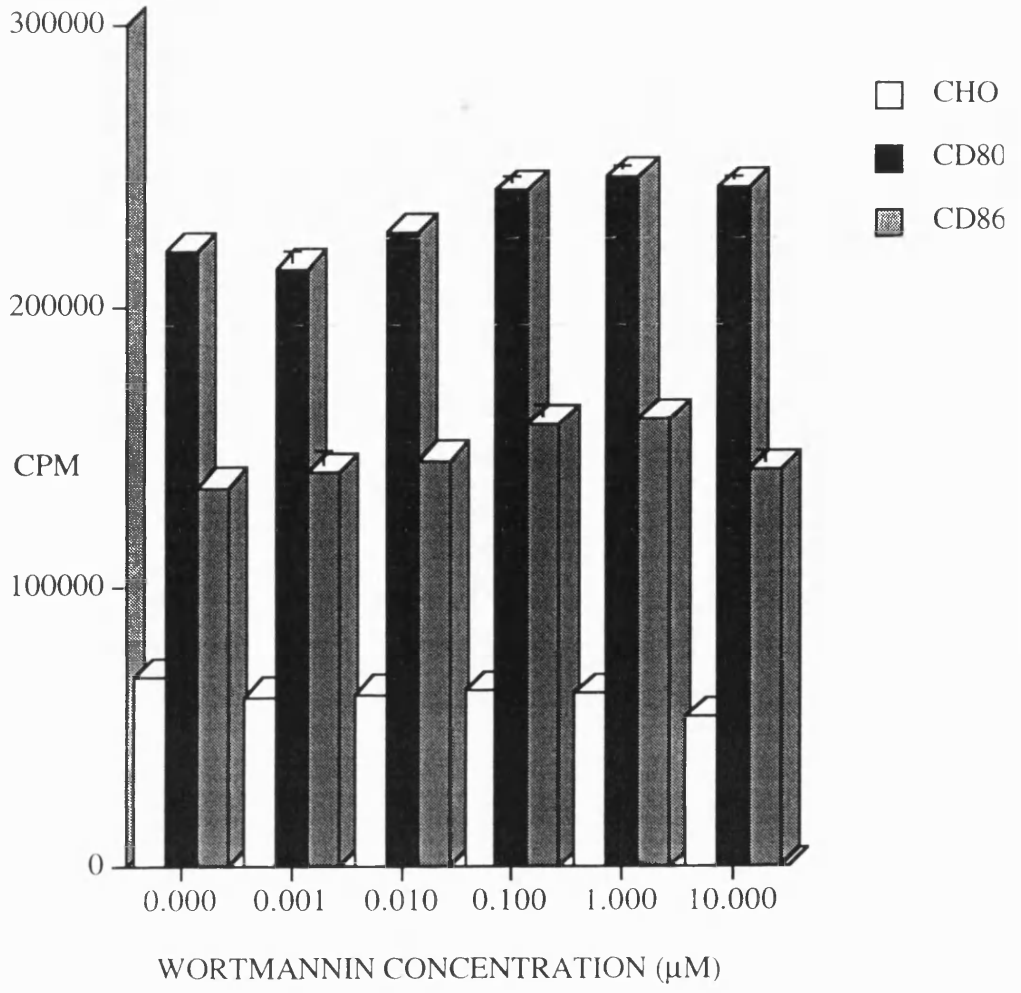


Figure 6.9 Effect of wortmannin on CD86 stimulation of T cell blasts.
5 x 10^4 T cell blasts (6 days post initial stimulation) were cultured with 1 x 10^4 transfectants as shown in increasing concentrations of wortmannin. Proliferation was measured after 48 hours using ^3H thymidine incorporation.The data are from triplicate samples from a single representative experiment (n = 5).

All the data generated in this research suggest multiple functions for CD28. Signals through CD28 appear to be essential for induction of IL-2 production and proliferation in resting T cells. By providing this costimulatory stimulus, CD28 is able to ensure the survival of the resting T cells which may undergo apoptosis as a result of TCR only signals or be rendered anergic. CD28 has also been shown to be important in sustaining an immune response by augmenting proliferation of activated T cell blasts. This sustaining of the immune response does not appear to require IL-2 production suggesting that the signals generated by CD28 vary depending on the activation state of the T cell. This variation in signalling by CD28 is also seen when the PI3-kinase inhibitor wortmannin is used. PI3-kinase activity can be inhibited in resting T cells activated via the TCR and CD28 resulting in blocking IL-2 production and T cell proliferation. However if T cells are already activated wortmannin has no effect on their ability to proliferate in the presence or absence of CD28 stimulation. This suggests that the signalling pathways used in activated T cell proliferation does not require PI3-kinase activity and that the signals generated by CD28 also do not require PI3-kinase.

Chapter 7

Final Discussion

During the course of this research CD28 has been shown to play an important role in both the induction of T cell proliferation and amplifying the proliferation of activated T cells. The initial results demonstrated clearly that costimulation through CD28 was essential for T cell activation whereas signalling through CD2 was unable to induce IL-2 production and T cell proliferation. Interestingly there did not appear to be any effect on T cell proliferation by CTLA-4. This CD28 homologue is known to be upregulated following T cell activation (Linsley et al., 1992a) and has a higher affinity for the CD28 counter-receptors CD80 and CD86 (Linsley et al., 1994). Research also suggests that ligation of CTLA-4 results in the generation of negative signals - i.e. inhibition of IL-2 production and T cell proliferation by arresting cell cycling rather than inducing apoptosis (Krummel and Allison 1996, Walunas et al., 1996). This would suggest that CTLA-4 should perhaps be involved in inhibiting the activation of resting T cells. This was not seen from the data as although CTLA-4 upregulation can occur within 24 hours of T cells being activated (Linsley et al., 1992a) T cell proliferation reached a maximum after 5 days (Figure 3.8) suggesting that there was no inhibition by CTLA-4. However the data on CTLA-4 signalling are from mouse studies and although there is 100% homology in the cytoplasmic domains of mouse and human CTLA-4 it has still to be demonstrated whether the signals generated by human CTLA-4 are inhibitory. More recent studies on CTLA-4 surface expression has shown that CTLA-4 is retained intracellularly and cycled to the cell surface and that surface expression of CTLA-4 is raised by increasing intracellular calcium levels (Linsley et al., 1996). TCR signalling is known to activate PLCγ-1 which results in the release on calcium from intracellular stores. However when CD28 signalling in mouse splenic T cells was examined calcium levels were not increased following CD28 ligation (Abe et al., 1995). Similarly, CD28 signalling in human resting T cells is thought to be CsA insensitive suggesting that calcium mobilisation is not involved(June et al., 1994). It may be that the levels of calcium generated in activating resting T cells is insufficient to induce adequate levels CTLA-4 surface expression and so negative regulation by CTLA-4 does not occur. This would make sense as it would be inefficient of the immune system to activate T cells in response to foreign antigen and as a consequence of this activation switch off the T cells possibly before a complete immune response is mounted.

However it may be that CTLA-4 plays a more important role in regulating an active immune response. This research has demonstrated that CD28 can

augment proliferation in activated T cells. Activated murine splenic T cells have been shown to induce increased intracellular calcium levels (Abe et al., 1995) and similarly signals generated by CD28 in activated human T cells are considered to be CsA sensitive suggesting calcium mobilisation is occurring (June et al., 1994). It is possible that CTLA-4 expression is upregulated rapidly on activated T cells in response to increased levels of calcium and therefore due to CTLA-4 having higher affinity for CD80 and CD86, CTLA-4 may play an important role in regulating the immune response by inhibiting continued T cell proliferation so allowing the immune response to quiesce.

Additionally, research has suggested that CD28 expression is downregulated in activated T cells in response to ligation and that the re-expressed CD28 is hyporesponsive to further stimulation (Linsley et al., 1993). This hyporesponsiveness may be a method of ensuring that signals generated through CTLA-4 in activated T cells predominate so arresting T cell proliferation. In contrast, the results from stimulating T cell blasts (Chapter 4) suggest that CD28 expressed on activated T cells is capable of responding to ligation by CD80 and CD86. However, the T cell blasts used in CD28 stimulation experiments were generated using mitogens and this may not involve CD28 ligation. Similarly, although downregulation of CD28 was examined in resting T cells activated through the TCR and CD28, the responsiveness of the re-expressed CD28 was not determined. It is important to assess whether the CD28 downregulation following activation of resting T cells results in re-expression of a hyporesponsive CD28 and this could be achieved by examining whether the activated T cells were responsive to further stimulation by CD80 or CD86. In this way it would be possible to demonstrate both the responsiveness of the re-expressed CD28 to further stimulation and whether the proliferation of T cells, activated through TCR and CD28, can be augmented by stimulation through CD28.

Augmentation of proliferation of T cells blasts appeared to be independent of IL-2 production. This cessation of IL-2 production was dramatic - large amounts of IL-2 were produced by Day 4 blasts (approximately 400IU/ml) and two days later none was detected (Figure 4.4). Although IL-2 production in resting T cells activated via the TCR and CD28 also shows a decline in IL-2 production by day 5 (Figure 3.8b), the sudden loss of IL-2 in T cell blasts suggests that this may be in response to a negative signal. It has been demonstrated that CTLA-4 signalling results in inhibition of IL-2 production (Krummel and Allison 1996, Walunas et al., 1996) and it may be that CTLA-4

is responsible for switching off IL-2 production. However, although there is no IL-2 production by older T cell blasts (day 6 or older), these T cells are still able to proliferate and this proliferation can be augmented by CD28 signalling. This is at odds with the data from mouse studies which suggested that CTLA-4 signalling also inhibited T cell proliferation by arresting the cell cycle (Krummel and Allison 1996, Walunas et al., 1996). It may be that the signals generated by CTLA-4 in human T cells are different from those seen in mouse and that although IL-2 production may be inhibited by CTLA-4 signalling in human T cells proliferation of activated T cells is not inhibited. The continued proliferation of the activated T cells in the absence of IL-2 may be in response to other cytokines or may be due to signalling pathways which are independent of IL-2. The possible role of other cytokines in the augmented proliferation of T cell blasts could be examined using supernatant transfer assays. Nevertheless, proliferation of T cell blasts has been shown to decline with time. It is possible that the signals responsible for the continued proliferation of the T cell blasts decay or perhaps the inhibitory effects of CTLA-4 signalling on proliferation are effective at this time.

The role of CTLA-4 in the regulation of T cell activation is still being investigated and although there is evidence for negative regulatory effects of CTLA-4 signalling in mice it remains to be determined whether CTLA-4 signalling effects are the same in human T cells. Nevertheless, the importance of the regulatory role of CTLA-4 has been demonstrated in CTLA-4 knockout mice where lack of CTLA-4 has been shown to be lethal (Waterhouse et al., 1995). The mice exhibited a lymphoproliferative disorder with marked infiltration of activated T cell blasts into many major organs which resulted in early death. The CTLA-4 knockout mice demonstrate the importance of CTLA-4 to act as a negative regulator of T cell activation.

Blocking T cell activation may be a method of inhibiting inappropriate immune responses in transplant patients and in treatment of autoimmune diseases. There have been a number of studies carried out using the chimeric molecule CTLA-4-Ig to block T cell activation in both transplants in mice and murine models of autoimmune disease with startling results. When mice were treated *in vivo* with human CTLA-4-Ig rejection of xenogeneic (human) pancreatic islet cells was blocked and this inhibition was due to blocking CD80 costimulation of T cells and therefore T cell activation (Lenschow et al., 1992). The use of CTLA-4-Ig to prevent the rejection of transplants also

resulted in long-term donor specific tolerance of the transplant. The normal therapy used to prevent transplant rejection relies on pan-immunosuppressive drugs. Although suppression of the immune system will help prevent rejection of the transplanted tissue it also makes the transplant recipient susceptible to infection from other sources. The transplant recipient is therefore immunocompromised and subsequent infections from other sources, which under normal circumstances would be cleared by the immune system, may have fatal consequences. It would appear from these results that CTLA-4-Ig may be a less hazardous method of prolonging transplant survival providing CTLA-4-Ig is itself not detrimental to the transplant recipient.

Research to examine the role of CTLA-4 in regulating autoimmune diseases using mAbs to CTLA-4 has shown that in mice with murine relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) the presence of CTLA-4 mAbs accelerated and exacerbated the disease. If the mice were treated with CTLA-4 mAbs during remission the treatment resulted in increased disease severity and a higher incidence of relapses (Karandikar et al., 1996). This data suggests that as blocking CTLA-4 responses with mAbs resulted in increased disease severity, CTLA-4 may play a major role in the regulation of autoimmune disease. As many autoimmune diseases are characterised by the presence of autoantibodies, a function of CTLA-4 may be to inhibit production of these potentially destructive autoantibodies. The use of a soluble form of human CTLA-4-Ig *in vivo* in mice (Linsley et al., 1992b) resulted in suppressed T cell dependant antibody responses to two immunogens - sheep erythrocytes and keyhole limpet hemocyanin. Unlike the transplant studies, the CTLA-4-Ig did not induce tolerance to the immunogens. However the immunosupression by CTLA-4 was prolonged and was shown to be associated with the continued presence of CTLA-4-Ig found in the serum. The continued presence of CTLA-4-Ig without any obvious deleterious effects on the mice suggests that CTLA-4 -Ig may be a potent immunosuppressive agent which has low toxicity, high specificity and is highly stable *in vivo*.

This immunosuppressive use of CTLA-4-Ig has also been demonstrated in the suppression of murine lupus - a murine model for the human autoimmune disease, systemic lupus erythematosus (SLE) (Finck et al., 1994). The mice used in this study spontaneously develop an autoimmune disease that closely resembles human SLE. Using a murine CTLA-4-Ig chimeric molecule, autoantibody production (a clinical symptom of the disease) was suppressed and the life of the mice was prolonged. This protective function of CTLA-4-

Ig was possible even at the most advanced stages of the disease. This data seem to suggest a therapeutic role for CTLA-4-Ig in autoimmune diseases. However this was not the case when CTLA-4-Ig was used to treat spontaneous autoimmune diabetes in the non-obese diabetes (NOD) mouse model. The disease is characterised by early islet inflammation (insulitis at 2 - 4 weeks) followed by overt diabetes (at 12 weeks or more) but not all animals become diabetic even if they have insulitis. Initial studies showed that CTLA-4-Ig or anti-CD86 mAbs blocked the development of full-blown diabetes but had little effect on primary insulitis (Lenschow et al., 1995). If early CD28-CD80/CD86 interactions were inhibited (by breeding CD28 knockout NOD mice) the disease was not inhibited. Instead an earlier and more severe insulitis was followed by increased incidence and severity of diabetes (Lenschow et al., 1996). This clearly demonstrated that simply blocking CD28 interactions would not necessarily result in inhibition of autoimmune disease. The treatment of normal NOD mice with CTLA-4-Ig also demonstrated this point. If the CTLA-4-Ig was administered early (at 2-4 weeks) or very late (at 10 weeks or later) it had no effect on the disease. This suggests that CD28 has a different role to play at different stages of the disease. It would appear from both the NOD mice and the CD28 knockout NOD mice that CD28 interactions are critical for disease progression after the development of insulitis and if CTLA-4-Ig is not administered shortly after this stage, full blown diabetes will develop. This was demonstrated by the inhibition of disease by administration of CTLA-4-Ig in 5 - 7 week old NOD mice.

Early blockade of CD28 interactions in the NOD mice resulted in expansion of T_h1 cells and the severity of the disease increased whereas later blockade of CD28 interactions blocked T_h1 expansion and the severity of disease is diminished. There is also evidence to suggest that if the balance of T cell responses is skewed towards T_h2 using anti-IFN-γ mAbs or IL-4 the development of the disease can be blocked (Debray-Sachs et al., 1991; Rapoport et al., 1993). This suggests that disease development may be inhibited by influencing the cytokine profile produced by T cells. This modulation of T_h1/T_h2 development and therefore the cytokines produced may play a role in therapies for a number of other diseases.

It has been shown that CD86 preferentially induces IL-4 production in activated T cells (Freeman et al., 1996) and as IL-4 is associated with the T_h2 subpopulation of T helper cells, it may be that CD86 stimulation results in the generation of T_h2 T cells whereas CD80 stimulation results in T_h1 cells being

generated. Although this research did not examine the cytokines produced by T cells following CD80 and CD86 stimulation, the data generated did suggest that CD80 and CD86 may play different roles in T cell activation. CD86 appeared to be more effective at costimulating resting T cells whereas CD80 stimulation of activated T cell blasts augmented proliferation more effectively. It may be that in the initial phase of T cell activation either CD28 ligand can stimulate T cells to produce IL-2 and proliferate. As CD86 is expressed on unactivated APCs, it is the ligand more readily available to initiate T cell activation. At this stage the T cells are expanding in number in response to IL-2 and differentiation into T_h1 and T_h2 cells may require additional signalling through CD28. As both CD80 and CD86 expression is upregulated following T cell activation (Hathcock et al., 1994) it may be that subsequent stimulation of activated T cells (as seen in Chapter 4) results in differentiation in to T_h1 and T_h2 T cells. Although it is still to be determined whether CD80 and CD86 stimulation of activated T cells results in the differential development of T_h1 and T_h2 T cells, there is evidence to support this potential role for CD80 and CD86.

Using blocking mAbs to CD80 and CD86 it has been demonstrated *in vivo* that treatment with anti-CD80 mAbs during immunisation of mice with proteolipid protein (which induces EAE) resulted in the generation of T_h2 cells resulting in reduced incidence of disease. In contrast, treatment with anti-CD86 mAbs resulted in generation of T_h1 T cells resulting in increased disease severity. The amelioration of disease in response to anti-CD80 mAbs could be inhibited by cotreatment with anti-IL-4 mAbs. This suggests that the initial secretion of IL-4 by T_h2 cells is an important feature of the protective effect of the anti-CD80 mAbs. Furthermore, adoptive transfer of the T_h2 cells prevented the establishment of disease and also abrogated already established disease (Kuchroo et al., 1995). Both the NOD mouse research and the EAE mouse data suggest that modulation of T cell differentiation and as a consequence the cytokine profile produced may be a potential therapy for autoimmune disease. However the NOD mouse data has also clearly demonstrated that timing the treatment is also of great importance. The research data is unclear as to whether the manipulation of the immune response in these mouse models of disease has had any effect on the ability of the mouse immune system to deal with other pathogens.

Although the role of CD80 and CD86 in modulating T cell differentiation has yet to be established, both CD28 ligands have been shown in this research to

be effective at costimulating resting T cells and stimulating activated T cells. However the differences in the effectiveness of CD80 and CD86 stimulation requires further investigation particularly as the magnitude of the differences between CD80 and CD86 varied depending on the T cell donor (Figures 5.2 and 5.5). The difference in proliferation levels generated between donors may be due to variation in the ratio of CD4 and CD8 T cells present. As only 50% of CD8 T cells express CD28 (Turka et al., 1990), T cells from one donor which have a higher ratio of CD8 T cell than T cells from another donor may have a lower level of proliferation as there is less CD28 available for stimulation. However this does not explain the difference in T cell proliferation in T cells from the same donor in response to CD80 and CD86 stimulation or explain why CD80 is more effective at stimulating T cell blasts and CD86 more effective at providing costimulation to resting T cells. Although binding assays have suggested that the affinity of CD80 and CD86 for CD28 is similar, the studies used chimeric CD28-Ig molecules (Linsley et al., 1994) and did not take into account that two CD80 and two CD86 molecules may bind a single CD28 molecule (Linsley 1995). Binding assays using surface iodination of T cells may help to clarify whether the differences in efficiency of stimulation by CD80 and CD86 is due to differences in binding to CD28. Since the efficiency of stimulation by CD80 and CD86 was different between resting and activated T cells, binding assays on both resting and activated T cells may determine whether affinity of CD28 for its ligands depends on the activation state of the T cell.

Although there are still may questions to be answered regarding the role of CD80 and CD86 in T cell activation, initial studies have determined a role for CD80 in the treatment of cancer. Using murine melanoma cells which express the E7 gene product of the human papilloma virus 16, the $E7^+$ tumour cells were found to grow progressively when injected into immunocompetant hosts. This demonstrated that the murine immune system was failing to respond to the presence of the human viral antigen expressed on the tumour cells. This may have been due to the lack of costimulatory molecules expressed on the surface of the melanoma cells. If the tumour cells were transfected with murine cDNA for CD80 (B7) and the resulting $E7^+B7^+$ tumour cells were injected into immunocompetant hosts, the tumour cells were rejected. The immune response was shown to be CD80 specific as it could be blocked by the addition of CTLA-4-Ig. A more startling result was seen when $E7^+B7^+$ cells were injected into mice with $E7^+B7^-$ tumours. The immune response generated in response to the $E7^+B7^+$ tumour cells also resulted in rejection of

$E7^+B7^-$ tumours at distant sites as well as resulting in regression of $E7^+B7^-$ metastases (Chen et al., 1992).

Similar results were obtained using another murine melanoma cell line to induce tumours in immunocompetant mice (Townsend and Allison, 1993). The melanoma cells although expressing MHC Class I and Class II molecules did not appear to provide the required costimulatory signals for T cell activation. If the melanoma cells were transfected with CD80 the tumour cells were rejected. It was possible to demonstrate that this rejection was mediated by CD8 cytotoxic T cells and that CD4 T cells were not required. Interestingly the rejection of the $CD80^+$ melanoma cells was found to protect a high percentage of the mice from subsequent challenge with $CD80^-$ melanoma cells. This suggested that the $CD80^+$ melanoma cells could effectively prime the immune response. The results of both these studies suggest that CD80 transfection may play a role in both the regression of existing tumours and also in priming the immune response to respond to the presence of tumourigenic cells in the future. It is interesting to note that in both studies tumour rejection was mediated by CD8 T cells in response to CD80 costimulation. It is possible that the results obtained in the mice models would not be as effective in humans due to the difference in expression of CD28 - all murine T cells express CD28 whereas only approximately 50% of human CD8 T cells express CD28. However if the transfection of CD80 into tumour cells can result in rejection of tumours (without any deleterious side effects) and reduce the need for chemotherapy and radiotherapy both of which have unpleasant side effects, its possible use as a therapy for cancer should be considered.

The research so far from animal models of disease has shown that immunotherapies based on immunomodulation of CD28 interactions may be the way forward to treating a number of incapacitating and sometimes potentially fatal diseases. However the same research has also highlighted the need for caution as the efficacy of a particular form of treatment will depend not only on the disease involved but may vary depending on the timing of the immunotherapy. It is therefore very important to increase our knowledge of the role of CD28 in normal immune responses to ensure that in any immunomodulation of CD28 interactions, the results obtained outweigh the possible risks generated.

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Appendices

Appendix 1.

Suppliers of equipment and consumables.

Amersham International plc
Amersham Place
Little Chalfon — Tel No: 01494 544000
Bucks
HP7 9NA

Appligene
Pinetree Centre
Durham Road
Britley — Tel No: 0191 4920022
Chester-le-Street
DH3 2TD

Becton Dickinson UK Ltd
Between Towns Road
Cowley — Tel No: 01865 748844
Oxford
OX4 3LY

Boehringer Mannheim
Bell Lane
Lewes — Tel No: 01273 480444
East Sussex
BN7 1LG

CP Pharmaceuticals Ltd
Wrexham
(Distributed via Fisons)

Dynal (UK) Ltd
10 Thursby Road
Croft Business Park
Bromborough — Tel No: 0151 3461234

Wirral
Fahrenheit Lab. Supplies
Unit 5,
Bridge Road
Kingswood — Tel No 0117 9701667
Bristol
BS15 4PW

Fisons Ltd
Loughborough
Leics
E11 0RG

Life Technologies
PO Box 35
3 Washington Road
Paisley — Tel No: 0141 8146100
Scotland
PA3 4EF

NBL Gene Sciences Ltd
South Nelson Industrial Estate
Cramlington — Tel No: 01670 732992
Northumberland
NE23 9HL

Nycomed (UK) Ltd
Nycomed House
2111 Coventry Road
Sheldon — Tel No: 0121 7422444
Birmingham

Pharmacia Biotech Ltd
23 Grosvenor Road
St Albans — Tel No: 01727 814000
Herts
AL1 3AW

Promega Ltd
Delta House
Enterprise Road
Chilworth Research Centre — Tel No: 01703 760225
Southampton
SO1 7NS

Sigma Aldrich Ltd
Fancy Road
Poole — Tel No: 01202 733114
Dorset

Skatron Ltd
Unit 11,
Studlands Park Ave.
Newmarket — Tel No: 01638 660600
Suffolk
CB8 7DB

Unipath Ltd
Basingstoke
Hants.

Wallac Ltd
P.O.Box 10
Turka
Finland
(Distributed through Fisons and Pharmacia Biotech)

Appendix 2

Recipes for culture media.

1. Glutamine - free medium for CHO transfectants.

To 400 ml of autoclaved distilled deionised water the following were added

Dulbeccos MEM (10 x)	55ml
Foetal Calf Serum (FCS)	50ml
Penicillin/streptomycin (10,000 IU/ml/10,000µg/ml)	5ml
Sodium bicarbonate (7.5%)	28ml
Sodium pyruvate (100mM)	5ml
Nucleosides (100x) (see below)	5ml

Nucleosides (100x)
0.24mg/ml thymidine
0.70mg/ml guanosine
0.70mg/ml adenosine
0.70 mg/ml cytidine
0.70mg/ml uridine

The nucleosides were added to 50ml of distilled deionised water, filter sterilised and stored at 4°C.

G418. Geneticin G418 - sulphate (100x)
50mg was dissolved in 1ml PBS which had been made up sterilely in autoclaved distilled and deionised water.

2. RPMI/10% FCS (complete medium)

RPMI 1640	400ml
FCS	50ml
Penicillin / streptomycin (10,000 IU/ml/10,000µg/ml)	5ml
L-glutamine (200mM)	5ml

This medium was used for culturing CTLLs and T cell blasts and in all proliferation assays.
Where media were required for washing cells and other non culturing requirements the following recipe was used

To 400 ml of autoclaved distilled deionised water the following were added

RPMI 1640 (10x)	50ml
FCS	50ml
Penicillin / streptomycin (10,000 IU/ml/10,000µg/ml)	5ml
L-glutamine (200mM)	5ml
Sodium bicarbonate (7.5%)	28ml

The medium was altered to pH 7.4 by the addition of 10N NaOH (0.8ml/500ml media).

Appendix 3

Recipes for Solutions /buffers used in mRNA determination.

Guanidinium thiocyanate (Stock solution).

		Final concentration.
Guanidinium thiocyanate	100g	4M
DEPC treated water	117ml	
0.75M sodium citrate pH7	7ml	25mM
10% sarcosyl	10.56ml	0.5%

Add the final three components to the guanidinium thiocyanate in the manufacturer's bottle and dissolve at 65°C. Store at 4°C.

To use for in RNA extraction -
add 72µl of 2-mercaptoethanol to 10ml of the stock solution.

Tris acetate EDTA (TAE 10X).
For 500ml of solution

Tris Base	24.22g
Sodium acetate	2.05g
Disodium dihydrate-EDTA	1.86g

add to deionised distilled water and pH to 8.1 with approximately 6 ml of glacial acetic acid.

Diethyl pyrocarbonate (DEPC)-treated water

Add 500µl of DEPC to 500ml distilled water , mix and leave overnight at room temperature in a Class 1 safety cabinet then autoclave.

DNA loading buffer (10X)

to 10ml of distilled water add		Final concentration.
bromophenol blue	25mg	0.25%
xylene cyanol	25mg	0.25%
Ficoll (type 400)	2.5g	25%

Store at room temperature.

RNA loading buffer (10X).

To 5ml of DEPC-treated water add		Final concentration
bromophenol blue	10mg	0.2%
Ficoll (type 400)	1g	20%

Store at room temperature.

Appendix 4

ELISATest for analysis of mAb binding.

The 96-well flat bottom tissue culture plate was coated with OKT3 at 1μg/ml for the required time periods (2, 4, 8, 24 hours). At each time point the appropriate wells were aspirated and washed with 250μl PBS, aspirated and then left covered with 250μl PBS until the whole plate was ready for testing using the ELISA kit (Supplied by Boehringer Mannheim).

The wells were aspirated and 100μl/well of 2%v/v FCS/PBS added and the plate incubated at 37°C for 1 hour.
The FCS/PBS block was aspirated and 100μl of the enzyme linked antibody added (anti- mouse IgG alkaline phosphatase diluted 1:999 in 2% v/v FCS/PBS). The plate was incubated for 1 hour at 37°C.
The wells were aspirated and washed (250μl/well each wash) with 2% FCS/PBS twice for 5 minutes each time and twice for 5 minutes with substrate buffer (0.5M sodium carbonate/bicarbonate pH9.6).
100μl/well of substrate (p-nitrophenyl phosphate (pNPP) at 1mg/ml in substrate buffer) was added and the plate incubated at 37°C for 30 minutes.
The plate was read on an ELISA Reader at 410nm.

Appendix 5.

Additional data.

Figure 4.2: CD80 augmentation of T cell blast proliferation over time.

Days	CHO	Std error	CD80	Std error	CD58	Std Error
4	217182.5	1797.8	272314.6	2818.5	233738.8	4882.8
6	88016.8	2896.6	258403.5	5700.4	86077.6	1747.4
8	89029.4	4629.5	213257.2	13861.7	63312.9	5080.2
10	80261.3	4093.3	156286.5	2031.9	61458.2	3761.2

Days	CHO	Std error	CD80	Std error	CD58	Std Error
4	113382.3	4500.1	130178.6	740.7	111914.5	2718.4
6	58815.4	4960.5	93579.5	305.1	66959.2	2014.8
8	55045.9	4802.8	98145.9	1298.0	42714.3	1823.9
10	25641.4	1248.8	38036.1	1579.3	16391.3	1210.5

Proliferation data are shown as the mean of triplicate cultures in cpm.

Figure 4.4 : IL-2 production by T cell blasts

Days	CHO	Std error	CD80	Std error	CD58	Std Error
4	48.5	1.1	284.2	5.1	40.6	1.0
6	0	-	2.4	0.1	0	-
8	0	-	0	-	0	-
10	0	-	0	-	0	-

Days	CHO	Std error	CD80	Std error	CD58	Std Error
4	197.7	7.5	385.5	15.1	173.8	19.1
6	0	-	7.5	0.6	0	-
8	0	-	0	-	0	-
10	0	-	0	-	0	-

-

The IL-2 production data are shown as the mean of triplicate cultures in iu/ml.

Figure 5.4 : CD86 stimulation of T cell blasts over time.

Days	CHO	Std error	CD80	Std error	CD86	Std Error
4	174150.5	7575.5	258955	8470.4	243146.1	9886.3
6	109963.5	3248.3	293074.5	7186.1	215317.8	4080.3
8	76282.5	2704.2	266510.3	18820.9	191891.2	7831.1
10	65742.5	2370.8	126721.8	5334.9	104376.9	5970.4

Days	CHO	Std error	CD80	Std error	CD86	Std Error
4	105694.9	128.4	119814.3	2000.9	116271.9	3267.2
6	48208.8	3254.6	87058.2	168.9	78555.4	3619.8
8	57573.4	2476.8	87222.4	777.2	70104.6	3422.5
10	19952.6	1259.0	36756.3	2157.2	30051.9	1877.3

The proliferation data are shown as the mean of triplicate cultures in cpm.

Figure 5.6: IL-2 production by T cell blasts in response to CD86 stimulation.

Days	CHO	Std error	CD80	Std error	CD86	Std Error
4	63.7	1.2	267.2	4.9	102.7	8.7
6	0	-	10.3	1.0	0	-
8	0	-	0	-	0	-
10	0	-	0	-	0	-

Days	CHO	Std error	CD80	Std error	CD86	Std Error
4	203.9	8.1	403.4	9.7	281.9	10.7
6	1.2	0.1	11.8	1.2	3.5	0.3
8	0	-	0	-	0	-
10	0	-	0	-	0	-

The IL-2 production data are shown as the mean of triplicate cultures in iu/ml.

Figure 6.8 : Inhibition of T cell proliferation by wortmannin.

Wortmannin concemtration (μM)	OKT3/CD80	Std Error	OKT3/CD86	Std Error
0	99170.0	7931.6	197408.7	5987.4
0.0001	98639.2	7594.1	193627.1	5527.6
0.001	96815.2	7443.2	181579.4	5024.3
0.010	69960.3	3562.7	198213.1	19821.3
0.100	28276.4	675.5	199357.1	15106.1
1.0	5209.9	119.4	124820.7	9115.6
10.0	1459.0	79.4	32424.6	3383.5

Wortmannin concemtration (μM)	OKT3/CD80	Std Error	OKT3/CD86	Std Error
0	176778.9	12669.7	187280.5	5403.0
0.0001	170648.2	6571.7	192734.5	16712.5
0.001	168701.5	5816.8	208221.6	8678.0
0.010	152978.6	10090.5	129489.5	12796.3
0.100	93011.6	9132.8	96316.4	1504.8
1.0	45790.4	1793.9	63494.0	4648.8
10.0	1009.2	51.4	22471.2	1105.8

The proliferation data are shown as the mean of triplicate cultures in cpm.

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Inhibition of CD28-mediated T cell costimulation by the phosphoinositide 3-kinase inhibitor wortmannin*

T lymphocyte activation requires at least two signals, one via the antigen-specific T cell receptor and a second via the surface molecule CD28 which provides signals critical to interleukin-2 (IL-2) production and T cell proliferation. We have previously shown (Ward S. G., Westwick J., Hall N. and Sansom D. M. *Eur. J. Immunol.* 1993. *23:* 2572) that CD28 stimulates phosphoinositide (PI) 3-kinase activity, indicating that D-3 phosphoinositides may act as mediators of CD28-induced T cell costimulation. Here, we report that immunoprecipitation of CD28 molecules from Jurkat cells stimulated with the CD28-ligand B7, results in a ligand-dependent association of CD28 with PI 3-kinase. This association correlates with the appearance of PI 3-kinase enzymatic activity in CD28 immunoprecipitates and the formation of D-3 phosphoinositides. Consistent with the hypothesis that D-3 phosphoinositides are important mediators of CD28 signaling, treatment of T cells with the PI 3-kinase inhibitor wortmannin, inhibited both T cell proliferation and production of IL-2, but not the response of T cells to exogenous IL-2. Hence, abrogation of PI 3-kinase activity by wortmannin, appears sufficient to disrupt the costimulatory pathway utilized by CD28, indicating a central role for this enzyme in the CD28 signaling pathway.

1 Introduction

Activation of resting T lymphocytes requires at least two signals: one provided by engagement of the TcR by foreign antigen complexed with self-MHC and the second by a costimulatory molecule present on antigen presenting cells [1, 2]. The major costimulatory signal is believed to involve the CD28 antigen on T cells (reviewed in [2] and [3]) binding to its ligands B7-1 (also termed CD80, herein referred to as B7) or B7-2 (also termed B70) [3–6] on the antigen presenting cell. This interaction has been shown to control proliferation and IL-2 production from TcR-stimulated $CD28^+$ T cells [7, 8] and it has recently been shown that T lymphocytes derived from $CD28^{-/-}$ knockout mice exhibit major defects in responses to mitogens [9].

The nature of the intracellular events responsible for CD28-mediated costimulation is currently ill-defined (reviewed in [2]). However, we have previously shown that ligation of CD28 by B7 induced the formation of D-3 phosphoinositides in T lymphocytes [10]. Since these lipids are formed as a result of phosphoinositide (PI) 3-kinase activity, our findings suggested that CD28 activates the PI 3-kinase signaling pathway. PI 3-kinase phosphorylates the membrane lipid phosphatidylinositol, at the position 3 on the inositol ring, resulting in the generation of phosphatidylinositol-(3)-monophosphate [PtdIns(3)P], phosphatidylinositol-(3,4)-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol-(3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] [11]. These phosphoinositide lipid isoforms represent only a minor fraction of cellular phosphoinositides [11] but considerable importance has been attached to this pathway, particularly concerning their putative role as regulatory molecules (reviewed in [11] and [12]. Recent kinetic evidence has suggested that PtdIns(3,4,5)P_3 formed by a phosphatidylinositol-(4,5)-bisphosphate- [PtdIns(4,5)P_2] specific-3-kinase, is the probable physiological intracellular mediator and this is subsequently degraded by sequential dephosphorylation to PtdIns(3,4)P_2 and PtdIns(3)P (reviewed in [11]).

The PI 3-kinase enzyme is a heterodimer consisting of a 85-kDa regulatory subunit containing two *src*-homology (SH2) domains and an SH3 domain [13], tightly associated with a catalytic 110-kDa subunit [14]. Studies of the binding specificity of the p85 SH2 domains reveal a core phosphotyrosine-containing consensus motif, namely YXXM [15]. Analysis of the cytoplasmic domain of CD28 reveals such a motif (YMNM) around the tyrosine at position 173, suggesting a direct interaction between PI 3-kinase and CD28 [3]. However, the functional relevance of this association remains unclear. In this study, we have used the fungal metabolite wortmannin which has recently been described as a specific inhibitor of PI 3-kinase [16–18], to assess the importance of PI 3-kinase to CD28-mediated costimulation. These experiments demonstrate that CD28-mediated increases in D-3 phosphoinositides, but not the association between CD28 and PI 3-kinase following ligation by B7, can be specifically inhibited by wortmannin.

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Abbreviations: **PI 3-kinase:** Phosphoinositide 3-kinase **PtdIns:** Phosphatidylinositol **PtdIns(3)P:** Phosphatidylinositol-(3)-monophosphate **PtdIns(3,4)P_2:** Phosphatidylinositol-(3,4)-bisphosphate **PtdIns(3,4,5)P_3:** Phosphatidylinositol-(3,4,5)-trisphosphate **SH:** *src*-homology domain **CHO:** Chinese hamster ovary **TLC:** Thin-layer chromatography **$[Ca^{2+}]_i$:** Cytosolic free calcium concentration

Key words: CD28 / Phosphoinositide 3-kinase / Costimulation / Wortmannin

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Furthermore, wortmannin abrogates B7-dependent T cell proliferation, but has no effect on T cell proliferation elicited by other stimuli. These data demonstrate that recruitment and activation of PI 3-kinase by CD28 is of major importance in CD28-mediated T cell functions.

2 Materials and methods

2.1 Reagents and antibodies

CD28 mAb 9.3 was kindly provided by Carl June (Naval Medical Research Institute, Bethesda); CTLA4Ig fusion protein was obtained from P. Linsley (Bristol-Myers Squibb, Seattle); UCHT1 and p85 mAb were from Doreen Cantrell (ICRF, London); p85 rabbit polyclonal antisera was from Mike Waterfield (Ludwig Institute, London); human rIL-2 was a gift from I. Lindley, (Sandoz, Vienna). OKT3 was obtained from ATCC (Rockville, MD). Ionomycin and fura-2 acetoxymethyl ester were purchased from Calbiochem-Novachem (Nottingham, GB). Wortmannin, PMA and phosphoinositide lipids [soybean PtdIns and bovine PtdIns(4)P] were purchased from Sigma. Wortmannin was dissolved in ethyl acetate to a concentration of 20 mM and stored in aliquots at − 20 °C in the dark. Immediately prior to use, the stock was diluted in tissue culture medium to the concentrations shown in the figure legends. The final concentration of ethyl acetate was generally less than 0.05 %, a concentration that had no effect on any of the T cell responses. Ethyl acetate, in an equivalent concentration to the maximum wortmannin concentration in each experiment, was used as a vehicle control.

2.2 Cell culture

The leukaemic T cell line Jurkat, T lymphoblasts and the IL-2-dependent cell line CTLL were grown in RPMI-1640 supplemented with 10 % FCS, streptomycin (50 μg/ml) and penicillin (50 U/ml) at 37 °C [19, 20]. CTLL cultures were also supplemented with 20 IU/ml IL-2. Parental CHO cells (CHO-B7$^-$) and CHO cells transfected with B7 cDNA (CHO-B7$^+$) were established and maintained as previously described [21].

2.3 T cell purification and proliferation

Heparinized blood samples were separated on a histopaque (1.077) density gradient. Peripheral blood mononuclear cells were removed from the gradient and purified T cells were obtained by negative selection as described [21]. T cells were stimulated as described in the figure legends and proliferation was measured at 72 h by adding 1 μCi of [^{3}H] thymidine/well (1 mCi/ml, 2 Ci/mmol, Amersham International, GB) for the last 18 h of culture, followed by harvesting cells onto Whatman GF/A filters and analysis by liquid scintillation counting. Where appropriate, T cell supernatants were harvested, and assayed for IL-2 activity using the CTLL IL-2-indicator cell line [20]. In exogenous IL-2 response assays, 6–8 day T lymphoblasts [19] were washed and cultured in IL-2 at the concentrations shown and proliferation determined as already described.

2.4 Cell lysis and immunoprecipitation

Jurkat cells (6×10^7; except where otherwise stated) were gently co-sedimented with 2×10^7 B7-expressing CHO cells in a volume of 1 ml at 200 g for 2 min, incubated at 37 °C in RPMI-1640 for the times indicated and the pellets lysed in 1 ml lysis buffer (1 % NP40, 100 mM NaCl, 20 mM Tris pH 7.4, 10 mM iodoacetamide, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate). Lysates were pre-cleared and immunoprecipitates performed for 2 h at 4 °C as described [22] using the following antibodies coupled to protein A-Sepharose beads (Pharmacia): CD28 mAb 9.3 (1 μg/ml lysate), p85 polyclonal rabbit antisera (1 μl/ml lysate).

2.5 *In vitro* lipid kinase assays

Immunoprecipitates were washed and subjected to *in vitro* lipid kinase assays as described [22] using a lipid mixture of 100 μl of 0.1 mg/ml PtdIns and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM Hepes, pH 7.0, 1 mM EDTA). The reaction was initiated by the addition of 20 μCi [γ-^{32}P] ATP (3000 Ci/mmol, DuPont-NEN, Stevenage, GB) and 100 μM ATP to the immunoprecipitates suspended in 80 μl kinase buffer. The reaction was terminated after 15 min and phospholipids were then separated by thin layer chromatography (TLC) [22].

2.6 Electrophoresis and immunoblotting

Immunoprecipitated samples for immunoblotting were electrophoresed on 7–17 % SDS-PAGE and transferred by electroblotting onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA) as described previously [22] using 10 mM Caps ([cyclohexylamino]propane-sulfonic acid) buffer, pH 11 for 20–24 h at 0.3 mA. Blots were probed with p85 mAb and proteins visualized by chemiluminescence detection system (ECL, Amersham) with a sheep anti-mouse Ig conjugated with horseradish peroxidase as a secondary antibody (Amersham).

2.7 D-3 phosphoinositide labeling, extraction and HPLC separation

Jurkat cells (4×10^8) were labeled with 1 mCi [^{32}P] orthophosphate [8500–9120 Ci/mmol, DuPont-NEN] as described [10, 23]. ^{32}P-labeled Jurkat cells were stimulated as described in the figure legends and phospholipids were extracted with 750 μl of chloroform:methanol:H_2O (32.6:65.3:2.1 %, v/v/v, respectively) as described [24, 25]. The samples were deacylated and analyzed by anion exchange high performance liquid chromatography (HPLC) analysis using a Partisphere SAX column (Whatman) [10, 26]. The eluate was fed into a Canberra Packard A-500 Flo-One on-line radiodetector and the results analyzed by the Flo-one data program (Radiomatic, USA) [10]. Similarly, [^{32}P] PtdInsP which had been produced, separated and visualized in the *in vitro* lipid kinase assays, was recovered and collected into 2 ml Eppendorf tubes, deacylated and further separated by this HPLC method.

Eluted peaks were compared to retention times for standards prepared from [^{3}H] phosphoinositides (Amersham) and ^{32}P-labeled D-3 phosphoinositides as described elsewhere [10, 22].

2.8 Determination of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in Jurkat cells

Jurkat cells were suspended at 10^7 cells/ml in RPMI-1640 medium supplemented with 10% FCS and incubated for 30 min at 37 °C with 2.5 μM fura-2 acetoxymethyl ester as described previously [27]. The fluorescence of a 2 ml cellular suspension was monitored with a Photon Technology International Delta Scan Fluorimeter (dual excitation λ 340 nm and 380 nm, single emission λ 510 nm) at 37 °C. $[Ca^{2+}]_i$ was determined by fluorescence using Photon Technology International software program (South Brunswick, NJ, USA).

3 Results

3.1 An *in vitro* lipid kinase activity associates with CD28 immunoprecipitates

Experiments were carried out to determine whether ligation by B7 could promote association of CD28 with PI 3-kinase. Following co-sedimentation of Jurkat cells with CHO-B7$^+$ cells, CD28 immunoprecipitates were assayed to detect lipid kinase activity co-associated with CD28 following stimulation. This approach revealed that a phosphoinositide lipid kinase activity responsible for the formation of [^{32}P] PtdInsP was present in CD28 immunoprecipitates and that the appearance of this activity was dependent on stimulation by B7 (Fig. 1a, lanes 1–4). Lipid kinase activity was not observed in unstimulated Jurkat cells (Fig. 1a, lane 5), Jurkat cells co-sedimented with CHO-B7$^-$ cells (Fig. 1a, lane 6) or in control CD28 immunoprecipitates derived from CHO-B7$^-$ (Fig. 1a, lane 7). In addition, the lipid kinase activity was not present in immunoprecipitates derived from Jurkat cells co-sedimented with CHO-B7$^+$ that had been pre-incubated for 10 min with CTLA4Ig fusion protein to block the B7/CD28 interaction (data not shown).

The lipid kinase activity associated with CD28 immunoprecipitates (Fig. 1b, lanes 1 and 2) was inhibited by the PI 3-kinase inhibitor wortmannin at a concentration of 100 nM. This concentration of wortmannin also inhibited PI 3-kinase activity immunoprecipitated by p85 polyclonal antisera (Fig. 1b, lanes 3 and 4) but did not affect lipid kinase activity associated with total Jurkat cell lysates (Fig. 1b, lanes 5 and 6). Immunoblotting of CD28 immunoprecipitates with p85 mAb confirmed that the p85 subunit of PI 3-kinase was coprecipitated with CD28 following ligation by B7 (Fig. 1c, lanes 2–4) but was absent from CD28 immunoprecipitates derived from resting Jurkat cells (Fig. 1c, lane 1) as well as CHO-B7$^-$ and CHO-B7$^+$ cells (Fig. 1c, lanes 5–6). To confirm that the phosphoinositide lipid kinase activity was attributable to PI 3-kinase activity, the phosphorylated lipids were recovered and separated by anion exchange HPLC (Fig. 2a,b and c). This analysis showed unequivocally, that the lipid kinase activity associated with B7-activated CD28 molecules was indeed PI 3-kinase, whilst that present in lysates was predominantly PtdIns 4-kinase.

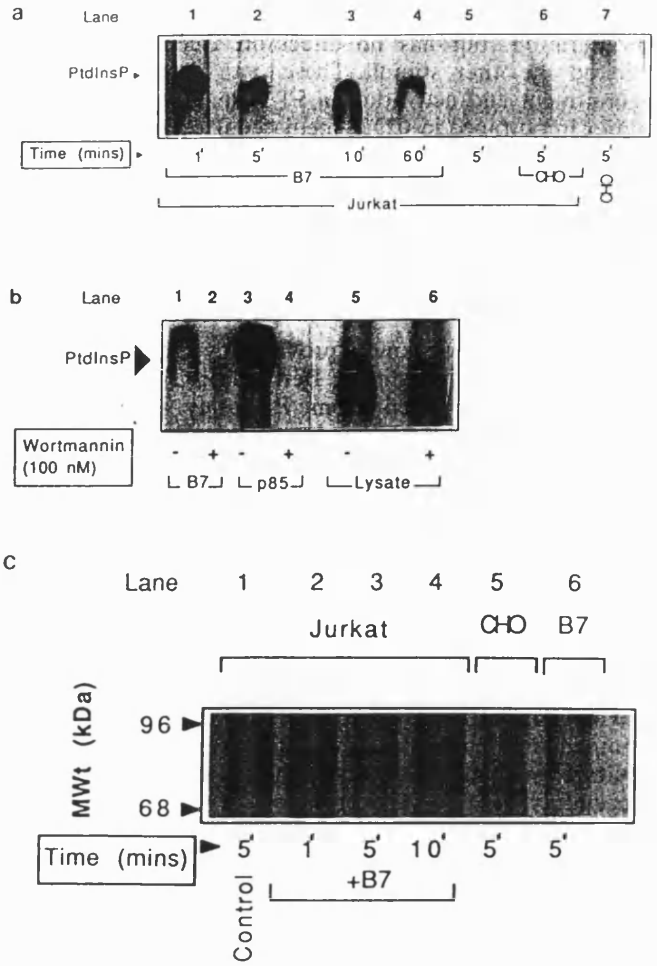

Figure 1. CD28 immunoprecipitates co-precipitate an *in vitro* lipid kinase activity. (a) Jurkat cells (6×10^7) were sedimented alone (lane 5), with CHO-B7$^+$ cells (2×10^7, lanes 1–4) or with parental CHO-B7$^-$ cells (2×10^7, lane 6). Parental CHO-B7$^-$ cells were also sedimented (2×10^7) as a control (lane 7). (b) Jurkat cells (6×10^7) were sedimented alone (lanes 3–6), or whith CHO-B7$^+$ (2×10^7), lanes 1–2). All lysates (a, lanes 1–6 and b, lanes 1 and 2) were subjected to immunoprecipitation with CD28 mAb. In addition, (b) resting Jurkat were lysed and immunoprecipitated with p85 polyclonal antisera (lane 3 and 4). (b) The washed immunoprecipitates, as well as 40 μl of total resting Jurkat cell lysate (lanes 5 and 6) were divided into two and analyzed for PtdIns kinase activity in the presence (lanes 2, 4, 6) and absence (lanes 1, 3, 5) of 100 nM wortmannin using PtdIns as a substrate. Extraction and separation of the ^{32}P-labeled phosphoinositide lipid products was performed as described in Sect. 2.7. (c) Jurkat cells (3×10^7) were sedimented alone (lane 1), or with CHO-B7$^+$ (1.5×10^7), lanes 2–4) for the times indicated. CHO-B7$^-$ cells (lane 5) and parental CHO-B7$^+$ cells (lane 6) were also sedimented (1.5×10^7 cells) as a control. Cells were lysed and all lysates were subjected to immunoprecipitation with CD28 mAb. The immunoprecipitates were washed and proteins resolved by SDS-PAGE, immunoblotted with p85 mAb and proteins visualized with the ECL detection system as described in Sect. 2.6. Migration of the molecular mass standards is indicated to the left in kDa.

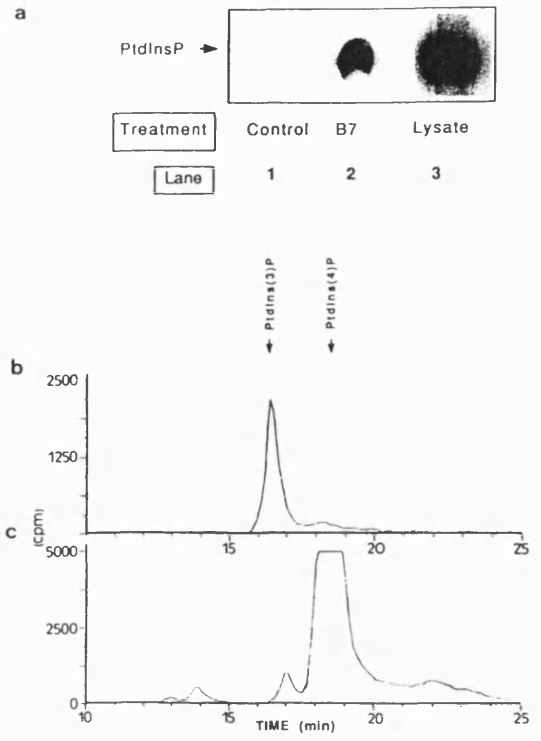

Figure 2. HPLC analysis of lipid kinase activity associated with CD28. (a) Jurkat cells (1×10^8) were sedimented alone (lane 1), or with CHO-B7$^+$ (5×10^7, lane 2). Cells were incubated at 37 °C for 5 min. All lysates (lanes 1 and 2) were subjected to immunoprecipitation with CD28 mAb. The washed immunoprecipitates and 40 µl of total resting Jurkat cell lysate (1×10^8 cells, lane 3) were analyzed for PtdIns kinase activity using PtdIns as a substrate. (b) The HPLC elution profile of the glycerophosphoinositol derivative of [^{32}P] PtdIns(3)P formed by CD28 immunoprecipitates derived from B7-activated Jurkat cells in panel a (lane 2). (c) HPLC elution profile of the glycerophosphoinositol derivative of [^{32}P] PtdInsP (predominantly [^{32}P] PtdIns(4)P) formed by Jurkat lysates in panel a, (lane 3). Extraction, TLC and HPLC separation of the lipid products was performed as described in Sect. 2.7.

3.2 Effect of wortmannin on CD28-mediated formation of D-3 phosphoinositides and $[Ca^{2+}]_i$

Having established that CD28 directly associated with PI 3-kinase following B7 ligation, we sought to correlate this interaction with the modulation of D-3 phosphoinositide lipids in intact Jurkat cells. The experiment shown in Fig. 3 demonstrates that co-sedimentation of CHO-B7$^+$ with Jurkat cells resulted in formation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. However, 10-min pre-treatment of ^{32}P-labeled Jurkat cells with wortmannin resulted in the concentration-dependent inhibition of the CD28-mediated increase in both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 following ligation by B7 (Fig. 3a and 3b). The IC_{50} for inhibition of CD28-mediated formation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 was 10 ± 5 nM and 7 ± 5 nM ($n = 5$) respectively. Immunoblotting with p85 mAb revealed that wortmannin pre-treatment at concentrations that inhibited D-3 phosphoinositide formation, had no effect on the physical co-association of CD28 with PI 3-kinase following ligation by B7 (Fig. 3c, lanes 1–3). In addition, wortmannin had no effect on the elevation of $[Ca^{2+}]_i$ induced by cross-linked CD28 mAb 9.3 (Fig. 4) or by the CD3 mAb UCHT1 (data not shown). Furthermore, the decrease from resting levels

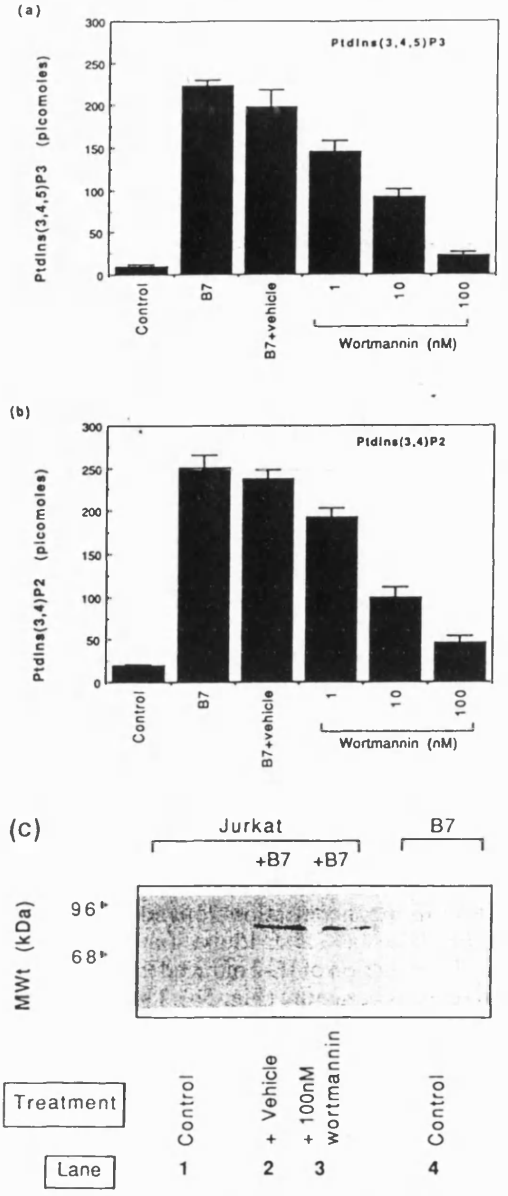

Figure 3. Wortmannin inhibits CD28-mediated formation of D-3 phosphoinositide lipids but not the association of CD28 with PI 3-kinase. ^{32}P-labeled Jurkat cells (3×10^7) were incubated for 10 min at 37 °C with vehicle or wortmannin (1–100 nM). Jurkat cells were then sedimented alone or with CHO-B7$^+$ cells (1.5×10^7) for 5 min. PtdIns(3,4,5)P_3 (a) and PtdIns(3,4)P_2 (b) were extracted, deacylated and analyzed by HPLC separation as described in Sect. 2.7. Data shown are mean ± SEM of at least four experiments. (c) Jurkat cells (3×10^7) were incubated for 10 min alone (lane 1), with vehicle (lane 2) or with 100 nM wortmannin (lane 3) at 37 °C. The Jurkat cells were then sedimented alone (lane 1), or with CHO-B7$^+$ cells (1.5×10^7, lanes 2–3). CHO-B7$^+$ cells were also sedimented (1.5×10^7) as a control (lane 4). Cells were lysed and all lysates were subjected to immunoprecipitation with CD28 mAb (lanes 1–4). The immunoprecipitates were washed, proteins resolved by SDS-PAGE under reducing conditions and immunoblotted with p85 mAb as described in Sect. 2.6. Migration of the molecular mass standards is indicated to the left in kDa.

of PtdIns(4,5)P_2 (an indication of phospholipase C activation) induced by UCHT1 [26] was also unaffected by wortmannin (data not shown).

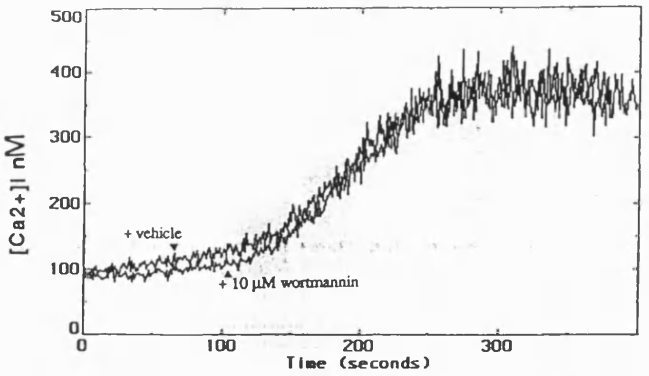

Figure 4. Effect of wortmannin on elevation of $[Ca^{2+}]_i$ induced by CD28 mAb 9.3. Fura-2 loaded Jurkat cells were incubated with vehicle or 10 μM wortmannin for 10 min prior to the addition of 10 μg/ml CD28 mAb 9.3 and goat anti-mouse cross-linking Ig (10 μg/ml). The trace is a recording of $[Ca^{2+}]_i$ as determined by the Photon Technology International software program. Data is from a single experiment representative of six others.

3.3 Wortmannin inhibits CD28-mediated costimulation of T cell proliferation and IL-2 production

Since wortmannin clearly inhibited the activity of PI 3-kinase in Jurkat cells, we next determined whether this compound could affect CD28-dependent costimulation of normal T cells. These experiments were performed using purified peripheral blood T cells which were stimulated to proliferate using a combination of the CD3 mAb OKT3 and CHO-B7$^+$ cells [21]. In this system, wortmannin inhibited T cell proliferation in a concentration-dependent manner (Fig. 5a) with an IC_{50} of 2.5 ± 15 nM ($n = 5$). More importantly, T cell production of IL-2 induced in this system was also abrogated by wortmannin (Fig. 5b). This suggested that the lack of T cell proliferation in this system was not due to a block in the T cell response to endogenous IL-2 but rather, due to a lack of IL-2 production following CD28 costimulation. The effect of wortmannin on other aspects of T cell function, namely purified T cell proliferation induced by PMA and ionomycin (Fig. 5c) and IL-2-driven proliferation of T lymphoblasts (Fig. 5d) was also measured. Surprisingly, there was no inhibition of proliferation due to the presence of wortmannin in either of these assays. Whilst performing these experiments, it was noted that incubation of wortmannin for 1 h in cell culture media prior to the addition of T cells, resulted in loss of inhibition by wortmannin of OKT3/CHO-B7$^+$-induced T cell proliferation (Fig. 5e). This correlated with the loss of inhibition by wortmannin of PI 3-kinase activity in p85 and CD28 immunoprecipitates as determined by *in vitro* lipid kinase assays (data not shown). These *in vitro* lipid kinase assays revealed that the inhibitory action of wortmannin on PI 3-kinase was unstable and abolished after as little as 30-min incubation of wortmannin with cell culture medium at 37 °C.

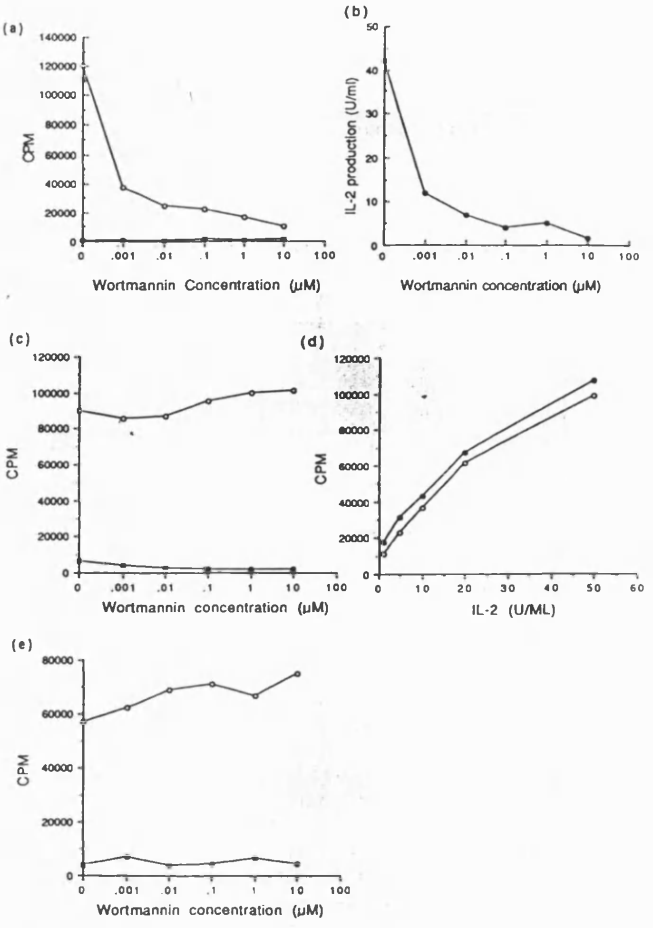

Figure 5. Effect of wortmannin on T cell proliferation and IL-2 production. Purified T cells (5×10^4) (a, b, c and e) were pre-incubated for 10 min with wortmannin (0.001–10 μM). Cells were stimulated with: (a and e) 1 μg/ml OKT3 plus 2×10^4 CHO-B7$^+$ cells (○), or 1 μg/ml OKT3 plus parental 2×10^4 CHO-B7$^-$ cells (●); (b) 1 μg/ml OKT3 plus 2×10^4 CHO-B7$^+$ cells; (c) 100 ng/ml PMA and 500 ng/ml ionomycin (○) or vehicle (●). (b) Supernatants from T cells stimulated as described were collected at 72 h. These supernatants were assayed for IL-2 production by incubation with 5×10^4 CTLL for 24 h and comparison with IL-2 standards. CTLL proliferative responses were determined following addition of [^{3}H] thymidine for the final 4 h of incubation as described in Sect. 2.3 (d) IL-2-dependent T lymphoblasts (1×10^5) were pre-incubated for 10 min with vehicle (●) or 10 μM wortmannin (○) followed by the addition of 1–50 U/ml IL-2. (e) Wortmannin was incubated for 1 h at 37 °C in cell culture medium followed by the addition of purified T cells and the appropriate stimuli as stated. Purified T cells (a, c, and e) and T lymphoblasts (d) were harvested at 72 h after pulsing with [^{3}H] thymidine as described in Sect. 2.3. The data represents triplicate samples from a single experiment representative of at least three others.

4 Discussion

The data presented in this report demonstrate the activation of PI 3-kinase following CD28 stimulation and the inhibition of this pathway by the PI 3-kinase inhibitor wortmannin. Our experiments are in agreement with previous reports [28–32] of a direct and rapid association of CD28 with PI 3-kinase following stimulation. The association between CD28 and PI 3-kinase appears to involve interactions between the YXXM motif and both the p85 N- and C-terminal SH2 domains [29, 31]. Since SH2 domains have specificity for phosphotyrosine residues [12, 15], this implies that phosphorylation of the CD28 molecule following ligation by B7 must occur, at least in part, on tyrosine 173. Accordingly, several groups using anti-phosphotyrosine mAb have detected tyrosine phosphorylation of immunoprecipitated CD28 following ligation [31, 32].

The major outstanding question regarding the CD28 association with and activation of PI 3-kinase, is the biological relevance of this interaction. To address this question, we have used the PI 3-kinase inhibitor wortmannin. This compound, whilst reportedly non-specific at micromolar concentrations [16–18], has been shown to specifically inhibit PI 3-kinase at nanomolar concentrations in numerous cells [16–18]. Accordingly, wortmannin inhibited the PI 3-kinase activity associated with p85 immunoprecipitates as well as the increases of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 following ligation of CD28 by B7. However, wortmannin did not affect PtdIns 4-kinase activity present in Jurkat cell lysates. Moreover, micromolar concentrations of wortmannin did not affect activation of phospholipase C and calcium mobilization mechanisms as determined by the lack of effect of wortmannin on UCHT1-induced PtdIns(4,5)P_2 metabolism as well as UCHT1- and CD28-induced $[Ca^{2+}]_i$ elevation.

Having established that wortmannin was indeed able to inhibit PI 3-kinase in T cells, the effects of this compound in assays of CD28-dependent T cell function were determined using naive T cells, activated T lymphoblasts and Jurkat cells. CD28 was found to associate with PI 3-kinase in naive T cells and T lymphoblasts after ligation by B7, in a similar manner to that reported in Jurkat (unpublished observations). As such, these T cells provide a system with which to analyze the functional significance of CD28 coupling to PI 3-kinase. In Jurkat cells, wortmannin inhibited the elevation of D-3 phosphoinositides following CD28 activation and this action may be responsible for the potent inhibition by wortmannin of B7-dependent T cell proliferation. The observed effects of wortmannin do not appear to be due to toxic effects of wortmannin, since incubation with wortmannin had no effect on cell viability (unpublished observations) and high concentrations of wortmannin did not abolish T cell proliferation induced by phorbol esters and ionomycin. It is significant that the B7-induced co-association of PI 3-kinase with CD28 was unaffected by wortmannin pre-treatment, such that the effect of wortmannin on CD28 signalling would appear to be primarily on the enzymatic activity of PI 3-kinase and not the ability of CD28 to recruit PI 3-kinase. Indeed, the site of action of wortmannin is believed to be the ATP binding site on the p110 catalytic subunit [17].

The observation that wortmannin abrogated IL-2 production induced by CD28 ligation is an important finding. In the primary T cell system used in this study, it has previously been demonstrated [8, 21] that the regulation of IL-2 production and proliferation are both functions of CD28 costimulation. Clearly, the abrogation of IL-2 production by wortmannin is highly consistent with a primary blockade of the CD28 signaling pathway and not later events such as responses mediated by IL-2. The lack of IL-2 production as determined using the IL-2-dependent CTLL cell line, seems unlikely to be explained by a direct effect of wortmannin on the CTLL cultures, since our data indicates that the ability of wortmannin to inhibit PI 3-kinase as determined by *in vitro* lipid kinase assays, is completely abolished after approximately 30 min incubation under cell culture conditions.

Interestingly, certain T cell responses were insensitive to wortmannin. Firstly, wortmannin had no effect on the response of T lymphoblasts to exogenous IL-2. This was surprising in view of the fact that PI 3-kinase has previously been shown to associate with the IL-2 receptor [33] and D-3 phosphoinositides are elevated upon IL-2 stimulation of T cells [34]. These observations may be due to coupling of the IL-2 receptor to a recently described wortmannin-insensitive PI 3-kinase isoform [35]. Alternatively, these observations may also relate to the instability of wortmannin under culture conditions and the use of T lymphoblasts. Wortmannin is known to irreversibly inhibit PI 3-kinase [17] but it is inactivated rapidly during the first few hours of cell culture. T lymphoblasts are highly metabolically active which may eventually result in the *de novo* synthesis of new pools of PI 3-kinase that are no longer exposed to functional wortmannin. Thus, at the time at which proliferative responses to IL-2 are measured (72 h), wortmannin appears to have little effect. Further biochemical studies of IL-2 receptor signaling will be needed to address this issue. Another alternative is that the IL-2 receptor may be able to invoke multiple signaling pathways such that PI 3-kinase is not critical to IL-2 receptor signaling.

The second wortmannin-insensitive response was observed in PMA- and ionomycin-stimulated T cells. These experiments were performed in freshly isolated, purified, resting T cells which were not synthetically active and therefore *de novo* synthesis of PI 3-kinase may not explain this particular wortmannin-insensitive response. A possible explanation for this finding is that either PMA or ionomycin act individually or in combination, at a point distal to the generation of D-3 phosphoinositides and their subsequent target molecules. This is interesting since one candidate target for PtdIns(3,4,5)P_3 is the ζ isoform of PKC [36]. However, this enzyme has been reported to be phorbol ester insensitive [37].

In addition to CD28 [10] and the IL-2 receptor [33, 34], other T cell molecules such as the TcR and CD4 are known to associate with PI 3-kinase via interactions with the *src* family kinases *fyn* [38] and *lck* [39–41], respectively. Moreover, both the TcR [26] and CD7 [42] also induce D-3 phosphoinositide production following ligation, albeit at much reduced levels compared to CD28-mediated increases (unpublished observations). It is unclear therefore, why activation of this pathway, which is clearly coupled to and activated by multiple T cell surface receptors, should be a critical event in T cell costimulation when specifically activated by CD28. In this respect, heterogeneity of (i) PI 3-kinase isoforms [35, 43, 44], (ii) receptor coupling to PI 3-kinase (either tyrosine kinase-regulated or G protein-regulated mechanism) [43], (iii) PI 3-kinase substrate specificity [35], or (iv) magnitude of receptor-induced D-3 phosphoinositide formation, may determine distinct functions for different T cell surface receptors. This may explain the critical role of CD28-activated PI 3-kinase but not for instance, TcR-activated PI 3-kinase [26], at least with respect to T cell costimulation.

Observations from this and other recent studies have implicated PI 3-kinase as an important enzyme in CD28 function [10, 28–32]. The data presented here show that abrogation of the generation of D-3 phosphoinositides resulting from CD28 activation, prevents CD28-mediated costimulation and implies that the coupling of CD28 to PI 3-kinase and subsequent signaling represents a critical

physiological signal. Clearly, at some point signals generated via this pathway must converge with those generated by the TcR pathway in order for the observed potent synergy to occur. Recent evidence has demonstrated that the protein kinase JNK is a likely point at which signal integration occurs since simultaneous activation of TcR and CD28 results in JNK activation [45]. Interestingly, JNK is a novel member of the MAP kinase group [46], activation of which requires complex protein kinase cascades which have been postulated to involve regulation by PtdIns[3,4,5]P_3 [11]. However, the events distal to PI 3-kinase activation and ultimately D-3 phosphoinositide elevation remain unclear [11].

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B7/CD28 but not LFA-3/CD2 interactions can provide 'third-party' co-stimulation for human T-cell activation

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SUMMARY

The requirement for co-stimulation in T-cell activation has become firmly established, whilst the precise identity of the molecules involved remains uncertain. Some of the major co-stimulatory molecules include ICAM-1, LFA-3 and B7. We have investigated the abilities of both LFA-3 and B7 to co-stimulate T-cell proliferation under a number of conditions using transfected Chinese hamster ovary cells. Using anti-CD3 antibodies we observed that B7 but not LFA-3 transfectants were capable of co-stimulating proliferation in purified peripheral blood T cells. In addition, both LFA-3 and B7 could induce proliferation in response to phytohaemagglutinin (PHA) and we obtained additive effects using both B7 and LFA-3 together. Using the superantigen staphylococcal enterotoxin B (SEB), we observed that presentation to purified T cells required the presence of class II-positive transfectants and that sensitivity to antigen was increased approximately 100-fold by the co-transfection of either B7 or LFA-3. However, when co-stimulatory molecules were provided by cells separate from those engaging the T-cell receptor (TcR), only B7 was capable of enhancing proliferation. Kinetic studies which investigated the time dependence for co-stimulation revealed that T cells responding to anti-CD3 antibodies required the B7 co-stimulation within the first few hours, for proliferation to be effective. Our data differentiate between the co-stimulatory abilities of B7 and LFA-3 and support the concept of a pivotal role for B7 in T-cell proliferation.

INTRODUCTION

The activation of T lymphocytes within the immune system is of fundamental importance in generating both cell-mediated and humoral responses. Increasing evidence suggests that this process requires at least two signals, one delivered by the α/β T-cell receptor (TcR) as well as a second delivered by a co-stimulatory molecule.[1] The absence of co-stimulation during TcR engagement is increasingly implicated in leading to a state of unresponsiveness (clonal anergy)[2,3] or possibly to programmed cell death (apoptosis).[4,5] Evidence from murine systems suggests that T cells may be rescued from clonal anergy by the delivery of a co-stimulatory signal by a 'third-party' cell which cannot engage the TcR.[6] This situation demands that any potential candidates for such co-stimulatory function be able to deliver signals from a cell different to that providing TcR engagement. Although there are several molecules reported to possess co-stimulatory activity, most have not been tested on third-party cells. Recently, interest in co-stimulation has focused on the molecule B7 and its T-cell ligand CD28.

B7 is a 44,000–54,000 MW glycoprotein first identified on the surface of activated B cells by the antibody BB1[7] and subsequently found on monocytes,[8] dendritic cells[9] and some repeatedly activated T cells.[10] B7 binds to the T-cell surface molecule CD28 on the majority of T lymphocytes, and modulation of CD28 has important effects on T-cell stimulation, including the augmentation of proliferation with anti-CD3 antibodies, alloantigens and lectins.[11,12] The enhancement of these responses appears to be effected by an increase in production of interleukin-2 (IL-2) brought about by accumulation and stabilization of IL-2 mRNA and direct up-regulation of the IL-2 enhancer.[13–15] It has also recently been established that the pathway by which CD28 signals is independent of the TcR and results in tyrosine phosphorylation.[16] Whilst data have accumulated which suggest the potential involvement of B7/CD28 interactions as co-stimulators, there is little evidence as to whether this form of co-stimulation is consistent with the effects observed in reversing clonal anergy, in particular whether B7 is effective as a 'third-party' co-stimulator in T-cell activation. In this study we have used Chinese hamster ovary cell transfectants to test whether B7-expressing cells can provide effective co-stimulation to cells expressing a ligand for the T-cell receptor only. Our data show that B7/CD28 but not LFA-3/CD2 interactions can deliver co-stimulation from a separate cell to that engaging the TcR. In addition, this interaction is time dependent in that the delivery of the B7 co-stimulatory signal is only effective within the first 24 hr following TcR engagement.

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These results provide strong support for the role of B7 as a critical co-stimulator of T-cell activation, which may well act as a switch between activation and anergy.

MATERIALS AND METHODS

Transfectants

Chinese hamster ovary (CHO) K1 cells were transfected with human cDNAs, encoding LFA-3, B7 and HLA-DR A and DR B1*0401. The DR genes were established under the control of a human cytomegalovirus immediate early promoter in the vector pEE6 hCMV. Cassettes containing DR4 genes were then subcloned into the vector pSVLGS.1, containing glutamine synthetase (GS) as a selectable marker (these vectors were a gift from Dr C. Bebbington, Celltech, Slough, U.K.). This construct was transfected by calcium phosphate precipitation into CHO cells and the cells selected using the GS inhibitor L-methionine sulphoximine (Sigma, Poole, U.K.) at 1 mM. Cells expressing high levels of DR molecules were cloned by limiting dilution. Subsequent expression of accessory molecules was achieved using the pEE6 expression vector transfected into DR-positive clones by electroporation. Selection of LFA-3 was based on G418, 500 μg/ml, and for B7 hygromycin was used at 250 μg/ml. All transfectants were grown in Dulbecco's minimal essential medium (DMEM) without glutamine, containing 10% fetal calf serum (FCS) and antibiotics with appropriate selection. The transfectants were routinely screened and sorted for expression by FACS and levels of expression were comparable between transfectants.

Monoclonal antibodies

The following monoclonal antibodies (mAb) were used: L243 (anti-DR) and TS2/9 (anti-LFA-3), obtained from ATCC (Rockville, MD); BB1 (anti-B7), obtained from Dr E. Clark (University of Washington, WA); BU12 (CD19), obtained from Professor I. MacLennan (University of Birmingham, U.K.); UCHM1 (CD14) and UCHT1 (CD3), obtained from Professor P. Beverley (ICRF, London, U.K.); CD25 antibody, obtained from Becton Dickinson (Mountain View, CA). For FACS analysis antibodies were used as undiluted culture supernatants, except BB1 and BU12 which were used as ascites diluted 1/2000 and 1/1000, respectively. In stimulation assays UCHT1 was used as dialysed 50% ammonium sulphate precipitate diluted to 10 μg/ml.

Antigen presentation assays

Transfectants were trypsinized and washed in RPMI-1640 containing 10% FCS prior to use. The cells were resuspended in 1 ml of complete medium up to 10^7 per ml and treated with mitomycin (100 μg/ml) for 2 hr at 37° or fixed with 0·025% glutaraldehyde for 2 min after pulsing with staphylococcal enterotoxin B (SEB) for 3 hr. After washing twice the cells were plated in 96-well flat-bottomed plates at $2·5 \times 10^4$/well for use as antigen-presenting cells (APC). In the kinetic experiments T cells were purified as detailed below and added at 5×10^4/well with anti-CD3 antibody at 10 μg/ml with or without co-stimulation. B7-transfected CHO cells were glutaraldehyde fixed and added at 2×10^4/well as co-stimulators at the time-points indicated post-stimulation. Cells were pulsed at equivalent time-points and frozen until counting. All samples were assayed in triplicate with standard errors routinely less than 10%. The data shown are representative of at least three independent experiments.

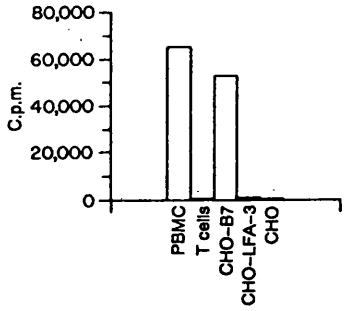

Figure 1. Co-stimulation of anti-CD3-induced activation by LFA-3 and B7 transfectants. UCHTI (10 μg/ml) induces proliferation in unseparated (PBMC) but not in purified T cells (T cells). The ability to proliferate is restored by B7 co-stimulation but not by LFA-3 or control CHO cells.

T-cell purification

Heparinized blood samples were separated on a histopaque (1·077) density gradient. Mononuclear cells recovered from the gradient were washed twice in phosphate-buffered saline (PBS) and used as responder cells without further fractionation at 10^5 cells/well. In experiments involving purified T cells, cells were isolated from peripheral blood mononuclear cells (PBMC) by negative selection. Briefly, after plastic adherence for 1 hr at 37° in FCS-containing medium, non-adherent cells were subjected to magnetic bead separation (Dynal-M450) using anti-DR (L243), anti-B cell (BU12) and anti-monocyte (UCHM1) antibodies. The resulting T cells were greater than 95% CD3 positive and used at 5×10^4/well with SEB or phytohaemagglutinin (PHA) at the concentrations shown. T-cell proliferation was measured at 72–96 hr by adding 1 μCi of tritiated thymidine/well for the last 8–24 hr of culture, followed by harvesting cells onto filters, and counting by liquid scintillation.

RESULTS

B7- but not LFA-3-expressing transfectants co-stimulate anti-CD3 antibodies

In the first series of experiments, B7- and LFA-3-expressing transfectants were tested for their ability to provide co-stimulation to purified resting peripheral blood T cells stimulated using anti-CD3 antibodies. It is known that in the absence of co-stimulators anti-CD3 is insufficient to induce proliferation. As shown in Fig. 1, unseparated PBMC responded vigorously to anti-CD3 antibodies added to the culture but the purification of T cells resulted in complete abrogation of the response. This loss of responsiveness is thought to reflect the removal of FcR-bearing cells which may cross-link the antibody but which also provide the co-stimulatory signals necessary for proliferation. Using this system we therefore investigated the ability of CHO transfectants expressing either B7 or LFA-3 molecules to provide this co-stimulatory requirement. This experiment (Fig. 1) demonstrated that B7 but not LFA-3 transfectants stimulated responses comparable to those obtained using unseparated cells. This confirmed that B7 is a major co-

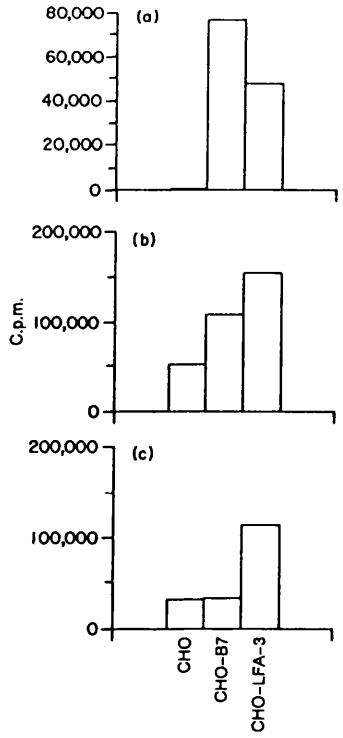

Figure 2. Co-stimulation of PHA-induced activation by LFA-3 and B7 transfectants. (a) PHA 1 μg/ml is incapable of inducing proliferation in purified T cells co-stimulated with CHO controls cells, whereas both B7 and LFA-3 transfectants induce a proliferative response. (b) PHA-treated T cells are responsive to exogenous IL-2 and both B7 and LFA-3 augment this response. (c) B7 and LFA-3 synergize in PHA responses. T cells were stimulated with PHA and B7 cells with the addition of further CHO, CHO-B7 or CHO-LFA-3 transfectants. This shows that the presence of both LFA-3 and B7 on separate cells results in increased responses to PHA.

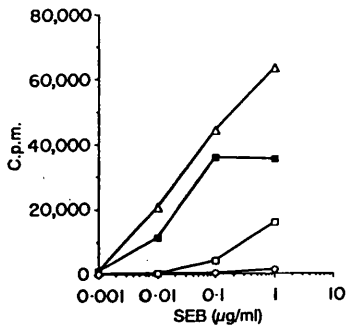

Figure 3. Effect of transfected accessory molecules LFA-3 and B7 on responses of purified T cells to SEB. Purified T cells are unable to respond to SEB in the absence of APC (○). However, responses were obtained by the addition of DR4 (□)-expressing CHO cells. DR4 cells co-transfected with either B7 (△) or LFA-3 (■) were used to assess the effect of these co-stimulatory molecules in response to a range of SEB concentrations.

stimulatory molecule in the induction of proliferative responses in resting T cells and demonstrated that LFA-3 was not effective in this situation.

Both LFA-3 and B7 can co-stimulate PHA-stimulated T cells

In order to further assess the abilities of both B7- and LFA-3-expressing transfectants in providing co-stimulation, the transfectants were added to T cells co-cultured with PHA as a stimulus. This experiment (Fig. 2) clearly demonstrated that, whilst purified T cells were incapable of responding to PHA alone, the addition of B7 and LFA-3 transfectants but not control CHO cells induced the T cells to proliferate strongly. Thus both the LFA-3 and B7 molecules expressed on the transfectants were functionally competent and capable of co-stimulation with PHA. In addition, the experiment was repeated with the addition of exogenous IL-2 to observe whether the PHA treatment had resulted in responsiveness to IL-2 in the absence of proliferation. The addition of IL-2 to the PHA-stimulated T cells (Fig. 2b) showed that the cells were responsive to IL-2 without further co-stimulation. Thus PHA stimulation of purified T cells allowed up-regulation of the IL-2 receptor but was insufficient to allow proliferation. However, where the T cells had been co-stimulated with PHA and either the B7 or LFA-3 transfectants, proliferation was further enhanced by IL-2 additions. In particular, responses to the LFA-3 transfectant were enhanced over three-fold, suggesting that signalling through CD2 might be affecting the level of expression of the IL-2 receptor. Since it is known that a major feature of CD28 engagement by B7 is an increase in IL-2 production we postulated that LFA-3 and B7 together would augment the response of purified T cells to PHA stimulation. As shown in Fig. 2c this proved to be the case. The addition of CHO or of further B7 cells to the PHA stimulation did not result in enhanced responses and indeed reduced the level of proliferation compared to optimized cell numbers. However, when both B7 and LFA-3 transfectants were added together they produced a considerable increase in proliferation despite supra-optimal numbers of transfectants being present. Together these data clearly demonstrate the ability of both B7 and LFA-3 to function as co-stimulators under different circumstances.

B7 but not LFA-3 transfectants provide 'third-party' co-stimulation of SEB-induced proliferation

Whilst the initial experiments established that B7 was a potent co-stimulator of T cells with anti-CD3, and that both B7 and LFA-3 molecules could co-stimulate PHA-induced proliferation, they did not allow us to conclude which of the interactions were required on the same surface as the TcR and which, if any, could take place through more remote interactions not involved in increasing adhesion between the T cell and its target. To address this issue, TcR and co-stimulatory signals were delivered separately to T cells by using transfectants expressing DR4 molecules coated with SEB as the TcR target and the co-stimulatory molecules B7 or LFA-3 as separate co-stimulators. Initially, experiments were performed to evaluate the ability of peripheral blood T cells to respond to SEB in the absence of APCs (Fig. 3). As expected, the purified T cells failed to respond, but the addition of transfectants expressing DR4 alone, DR4-LFA-3 or DR4-B7 double-transfectants demonstrated that

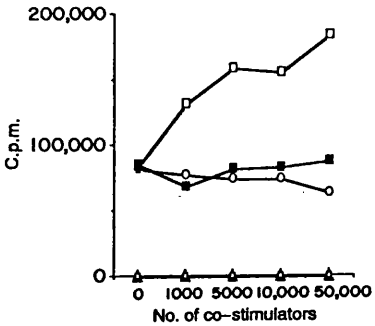

Figure 4. 'Third-party' co-stimulation is provided by B7 but not by LFA-3. DW4 transfectants were pulsed with 1 μg/ml SEB and used to stimulate purified T cells. Different numbers of CHO (○), CHO-LFA-3 (■) and CHO-B7 (□) cells were added to assess the ability of B7 and LFA-3 to provide co-stimulation from a 'third-party' cell. B7 (▲) and LFA-3 (□) single transfectants were used to control for background T-cell stimulation.

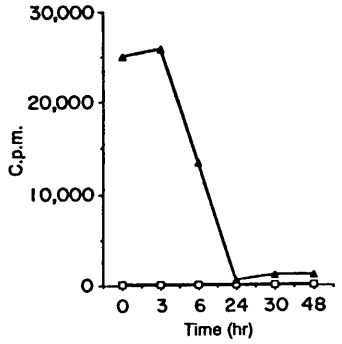

Figure 5. Kinetics of B7 co-stimulation with anti-CD3 antibodies. Purified T cells were stimulated with UCHT1 at 10 μg/ml. Fixed CHO (□) or CHO-B7 (▲) cells were added at times indicated after the start of the assay. Samples were harvested for counting after pulsing with tritiated thymidine overnight.

both LFA-3 and B7 could function as potent co-stimulators when present on the same cell as the TcR ligand. Subsequently, the experiments were repeated with B7 and LFA-3 co-stimulation provided by cells separate from those engaging the TcR (Fig. 4). In this situation only B7 proved capable of enhancing proliferation in resting T cells. This enhancement was dependent on TcR stimulation since B7 transfectants alone were unable to induce proliferation and thus clearly demonstrated co-stimulation by B7 but not LFA-3 on a third-party cell. These results are consistent with the observations that CD28 has a separate signalling pathway and that LFA-3/CD2 apparently requires CD3 stimulation for signalling.

The delivery of B7 co-stimulation is time dependent

Having demonstrated that B7 but not LFA-3 is capable of fulfilling a major requirement for co-stimulatory function, we next addressed whether the signal delivered by B7 was time dependent (as predicted by experiments capable of anergy prevention). We therefore used B7 or control CHO cells as co-stimulators of anti-CD3-induced proliferation and investigated the effects of delayed addition of the B7 co-stimulation. In these experiments purified T cells were treated with anti-CD3 at the start of the experiment and B7 or control CHO cells added at the times indicated (Fig. 5). The results from this experiment showed that for co-stimulation to be effective the signal must be delivered within the first 24 hr of TcR engagement. The addition of cytokines such as IL-1 and IL-6, as well as control CHO cells, in these experiments did not result in any co-stimulation.

DISCUSSION

The activation of T cells is thought to require at least two distinct signals, an antigen-specific signal delivered through the TcR (Signal 1), as well as a co-stimulatory signal (Signal 2) which enables the cell to proceed to IL-2 production and proliferation. A number of studies indicate that the delivery of Signal 1 in the absence of Signal 2 results not only in the absence of proliferation but also in a state of unresponsiveness with respect to the antigen providing Signal 1. This scenario is attractive also because it potentially explains a number of *in vivo* findings regarding tolerance.[17-19] However, to date the nature of the co-stimulatory signal(s) remains uncertain. Several molecules are known to provide some degree of co-stimulation for T-cell proliferation.[13,20-22] Of these, the best candidate appears to be B7 which has been shown in several studies to up-regulate the production of IL-2 by T cells. This cytokine has been suggested to play a key role in overcoming or preventing anergy. In this study we have established that B7 can co-stimulate T-cell proliferation in a way which is distinguishable from another important co-stimulatory molecule, LFA-3. In particular we have shown that B7 can fulfil the predictions for co-stimulatory function made by Jenkins *et al.*,[6] which include: (1) the ability to provide co-stimulation from a cell not providing Signal 1; (2) the ability to provide a signal which can synergize with CD3, resulting in proliferation; and (3) the ability to generate a signal which is time dependent in its ability to induce proliferation. The differences observed between B7 and LFA-3 do not appear to relate to levels of expression since these are comparable by FACS analysis. In addition, in functional analyses (using SEB) both B7 and LFA-3 molecules are capable of considerable functional effects.

Recently the ability of B7 to provide third-party co-stimulation has been addressed in the mouse by Liu and Janeway.[23] In this study, however, the authors concluded that third-party co-stimulation was limited when using anti-CD3 bound by FcR on one cell and providing co-stimulation via LPS-treated B cells or transfectants expressing B7. There may be several reasons for the discrepancies between our data. First, the mouse system may indeed be different in its ability to receive third-party co-stimulation. In this regard it is interesting that mouse T cells do not express class II molecules when activated, in contrast to human T cells which express both class II and B7 on some T-cell blasts. This may reflect a difference in the abilities of human and mouse T cells to receive B7 engagement of CD28 from other cells. Second, in our experiments we used SEB as an antigen for the TcR; this may have different co-stimulatory requirements to stimulation via CD3. It has recently been shown that stimulation via SEB may give different signals compared to normal antigenic stimulation.[24] Third, the levels of B7 expression on the co-stimulator cells may differ thereby affecting the ability of the third-party cells to co-stimulate. However, our

data are consistent with those of Liu and Janeway in other respects. We also find the expression of both ligands on the same cell to be a more potent stimulus for proliferation and that B7 is capable of direct stimulation of T cell blasts but not resting T cells (data not shown).

The ability of LFA-3 to co-stimulate PHA but not anti-CD3 antibodies is also an interesting finding. These experiments suggest that LFA-3 is not a co-stimulatory ligand for CD2 by itself and may require other signals from the same cell surface as the TcR. The fact that pairs of antibodies are required to stimulate through CD2 is consistent with this possibility.[25] In our experiments with SEB presentation by DR4-LFA-3 transfectants, LFA-3 may be serving to provide adhesion rather than co-stimulation, thereby providing increased sensitivity to antigen. Previously, however, we have noted, using SEA, that LFA-3 has the potential to enhance IFN-γ production, suggesting some signalling capacity although perhaps insufficient to generate a proliferative response.[26] It is also possible that CD2 stimulation rigorously requires the engagement of CD3 on the same surface, as would be provided by our DR4-LFA-3 co-transfectants, and that this results in an enhanced proliferative signal. In the experiments where PHA was present it appears that the lectin provides an additional ligand for CD2 which is capable of synergy with LFA-3, resulting in a more potent stimulus for T-cell proliferation. Recently, two new ligands for CD2 have been identified[27,28] and of particular interest is the report that mouse CD2 binds to the CD48/Blast-1 antigen. Given the restricted expression of the Blast-1 molecule on cells involved in the immune response,[29] this may provide the necessary signalling for proliferation in association with LFA-3.

Whilst our experiments have not directly tested the induction of anergy in peripheral blood T cells, it is clear that the use of anti-CD3 requires a co-stimulus to induce proliferation in these cells. Our data suggest that this co-stimulation can be provided by B7 but not LFA-3 and that the delivery of co-stimulation is restricted to the first few hours after TcR engagement. Clearly our data support the involvement of B7 as a major co-stimulatory molecule required for the proliferation of T cells and are consistent with a role in the prevention of T-cell anergy.

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Induction of Activator Protein (AP)-1 and Nuclear Factor-κB by CD28 Stimulation Involves Both Phosphatidylinositol 3-Kinase and Acidic Sphingomyelinase Signals[1]

Christine E. Edmead, Yusuf I. Patel, Anne Wilson, George Boulougouris, Nicolas D. Hall, Steven G. Ward, and David M. Sansom[2]

A major obstacle in understanding the signaling events that follow CD28 receptor ligation arises from the fact that CD28 acts as a costimulus to TCR engagement, making it difficult to assess the relative contribution of CD28 signals as distinct from those of the TCR. To overcome this problem, we have exploited the observation that activated human T cell blasts can be stimulated via the CD28 surface molecule in the absence of antigenic challenge; thus, we have been able to observe the response of normal T cells to CD28 activation in isolation. Using this system, we observed that CD28 stimulation by B7-transfected CHO cells induced a proliferative response in T cells that was not accompanied by measurable IL-2 production. However, subsequent analysis of transcription factor generation revealed that B7 stimulation induced both activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) complexes, but not NF-AT. In contrast, engagement of the TCR by class II MHC/superantigen, either with or without CD28 ligation, resulted in the induction of NF-AT, AP-1, and NF-κB as well as IL-2 production. Using selective inhibitors, we investigated the signaling pathways involved in the CD28-mediated induction of AP-1 and NF-κB. This revealed that NF-κB generation was sensitive to chloroquine, an inhibitor of acidic sphingomyelinase, but not to the phosphatidylinositol 3-kinase inhibitor, wortmannin. In contrast, AP-1 generation was inhibited by wortmannin and was also variably sensitive to chloroquine. These data suggest that in activated normal T cells, CD28-derived signals can stimulate proliferation at least in part via NF-κB and AP-1 generation, and that this response uses both acidic sphingomyelinase and phosphatidylinositol 3-kinase-linked pathways. *The Journal of Immunology,* **1996, 157: 3290–3297.**

Activation of T lymphocytes is central to the efficient functioning of the immune system. In addition to stimulation of the TCR by foreign Ags, the activation of resting T cells requires additional signals, which can be provided by the co-ordinated engagement of the CD28 molecule (1, 2). While CD28 has been known for some time to be a major costimulatory molecule, the signaling events associated with its engagement are still poorly characterized, especially in normal T cells. Recent progress has identified a number of receptor proximal events that appear to be triggered following ligation of CD28 either by its natural ligand B7-1 (CD80) or by activating anti-CD28 Abs. In particular, ligation of CD28 has been shown to result in the generation of 3′ phosphoinositides following the recruitment and activation of the enzyme PI3K[3] (3). This interaction is mediated via binding of the PI3K p85 subunit SH2 domains to the phosphorylated Tyr Met Asn Met (YMXM) motif in the cytoplasmic domain of CD28 (4). The importance of PI3K in CD28 signaling has been investigated by use of the 3-kinase inhibitor, wortmannin, which blocks the enzymatic activity of the kinase, but not its recruitment by CD28 (5). These studies have demonstrated that in normal resting T cells, wortmannin blocks both proliferation and IL-2 production in response to CD28 costimulation, but contrasts to the limited inhibitory effects seen using wortmannin in activated cells, such as Jurkat cells. Thus, the relative contributions of PI3K to CD28-induced proliferation and IL-2 production are currently unclear. One possible explanation for the difference in the effects of PI3K inhibition is the existence of alternative CD28-driven signaling pathways, which in activated cells may be capable of providing the necessary costimulatory signals. The recent discovery of the involvement of the acidic sphingomyelinase pathway in CD28 signaling (6) has, therefore, provided a second mechanism through which CD28 could exert its costimulatory actions.

The sphingomyelinase signaling pathway is a second messenger system that appears to be associated with a diverse range of surface receptors, including CD28, TNF (7–9), and Fas (10). Two different forms of the sphingomyelinase enzyme have been described: a membrane-bound neutral sphingomyelinase and an acidic form that is thought to be sequestered in lysosomes (11). Activation of the acidic form is sensitive to inhibition by lysosomotropic agents such as chloroquine (7), which affect lysosomal pH, and it is this acidic form that is activated by CD28. Activation of the sphingomyelinase enzyme results in the generation of phosphorylcholine and ceramide; the latter is the active signaling entity. Several downstream targets of ceramide have been identified to date, including activation of the Ras/Raf pathway (12), JNK (13), and PKCζ (14). PKCζ has been reported to be responsible for the induction of NF-κB via mechanisms involving phosphorylation and

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[3] Abbreviations used in this paper: PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor-κB; CHO, Chinese hamster ovary; AP-1, activator protein-1; SEA, staphylococcal enterotoxin A; CTLL, cytotoxic T lymphocyte line.

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subsequent degradation of IκB, the inhibitory subunit of the NF-κB complex (15). Degradation of IκB allows free translocation of the NF-κB to the nucleus, where it participates in gene regulation. The activation of acidic sphingomyelinase by CD28 ligation may, therefore, provide a link to the regulation of NF-κB.

While the downstream effects of CD28 signals are still under investigation, it is clear that a major outcome of CD28 costimulation is the regulation of cytokine genes at both the transcriptional and post-transcriptional levels (16, 17). Previous reports have suggested that CD28 may affect transcription factors that bind to the IL-2 and other cytokine promoters (18); however, many of these studies have been performed in Jurkat cells using anti-CD28 Abs in addition to mitogenic stimuli (such as PMA and ionomycin), making it difficult to interpret the precise effect of the CD28 stimulation.

In the present study, we have used normal activated T cells to investigate the regulation by CD28 of transcription factors known to bind to the IL-2 promoter. Exploiting the observation that superantigen-driven T cell blasts respond to CD28 stimulation in the absence of TCR engagement, we have used CHO transfectants expressing B7 to stimulate CD28 responses. Given that both PI3K and acidic sphingomyelinase pathways have been implicated in CD28 signaling, we have used this system to determine which transcription factors can be influenced by CD28 stimulation and which proximal signaling events influence their generation. Our results demonstrate that in activated T cells, CD28 ligation results in both proliferation and the induction of NF-κB and AP-1 complexes. Furthermore, using inhibitors of PI3K and acidic sphingomyelinase, we demonstrate that both of these signaling pathways are involved in the induction of these transcription factors by CD28.

Materials and Methods

Preparation of T cell blasts and purified T cells

Heparinized peripheral blood was diluted 1/1 with PBS, layered onto a Lymphoprep (Nycomed, Oslo, Norway) gradient (density, 1.077 g/ml), and centrifuged at 300 × *g* for 30 min. The mononuclear cells (PBMCs) were harvested and washed twice in medium. To generate T cell blasts, PBMCs were placed in a tissue culture flask at a density of 3 to 5 × 10^6/ml and stimulated with the superantigen SEA (10 ng/ml). The cells were then expanded in IL-2 as required. After 8 to 10 days, the blasts were restimulated with SEA Ag fixed onto DR4/B7 transfectants, and the cells were expanded in IL-2 for an additional 8 to 14 days before use.

Purified T cells were prepared as previously described (19). Briefly, PBMCs were subjected to plastic adherence for 1 h. Nonadherent cells were recovered and depleted by negative immunomagnetic selection of CD14-, CD19-, and HLA-DR-expressing cells. Cell purity was assessed by FACS, and populations were at least 95% $CD3^+$.

Transfectants

CHO cell transfectants were generated as previously described (19) by expressing the appropriate cDNAs downstream of a CMV promoter in pEE6 or pcDNA-3 expression vectors. Transfectants were cloned and sorted by FACS, and the expression levels of the relevant Ags were routinely monitored by FACS analysis. All the transfectant cells were cultured in glutamine-free DMEM containing 10% FCS and penicillin/streptomycin. For use in stimulation assays, transfectants were washed in PBS, fixed in 0.025% glutaraldehyde for 2 min, and extensively washed in complete medium before use.

Proliferation assays

Routinely, T cell proliferation in response to various stimuli was measured using 5 × 10^4 T cells/well in a flat-bottom 96-well plate in combination with 2 × 10^4 glutaraldehyde-fixed transfectants. Monoclonal Abs were added at 1 μg/ml in soluble form unless otherwise stated, and each treatment was conducted in triplicate. Assays were incubated for 72 h at 37°C. Aliquots of supernatant were then removed from each well and assayed for IL-2 production using a CTLL bioassay, as described previously (5). To measure proliferation, 1 μCi of [^{3}H]thymidine was added to each well, and incubation was conducted for an additional 8 h. After this time, the cells were harvested onto Whatman GFC glass fiber filters (Clifton, NJ), and the incorporation of radioactivity was assessed using liquid scintillation counting.

FACS analysis

The following Abs were used as staining reagents at 1 μg/ml: OKT11 (anti-CD2), UCHT1 (anti-CD3), E2H10 (anti-CD4), 8784 (anti-CD25), 9.3 (anti-CD28), and L243 (anti-HLA-DR). Staining was detected by the addition of FITC-conjugated anti-mouse polyvalent Igs (Sigma Chemical Co., St. Louis, MO), and 5000 cells were analyzed by flow cytometry on a Becton Dickinson FACStar Plus (Mountain View, CA). Abs against CD2, CD25, and DR were obtained from American Type Culture Collection (Rockville, MD), 9.3 was a gift from Dr. P. Linsley, and UCHT1 and E2H10 were obtained from Prof. P. Beverley.

Preparation of nuclear extracts

Cells (6 × 10^7) were washed, resuspended at 3 × 10^6/ml, and stimulated at 37°C for 4 h. Transfectants were used as stimulators at a ratio of 1:5 transfectants to T cells. For experiments involving enzyme inhibitors, the T cells were preincubated for 1 h before stimulation.

After stimulation, cells were washed in cold PBS, pelleted, and resuspended in 400 μl of hypotonic buffer 1 (10 mM HEPES, pH 7.8; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 0.2 mM PMSF; leupeptin E64; and pepstatin) supplemented with 0.2% Nonidet P-40 on ice for 15 min. Nuclei were pelleted by gentle centrifugation in a microcentrifuge. The pellet was gently washed in 1 ml of buffer 1 supplemented with 8.5% sucrose and recovered by centrifugation as described above. Nuclei were resuspended in 400 μl of buffer 2 (20 mM HEPES pH 7.8; 25% glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; and protease inhibitors as described above) and incubated on ice for 20 min. Lysates were centrifuged for 30 min, and ammonium sulfate (0.33 g/ml) was added to precipitate proteins. The protein pellet was resuspended in 50 μl of buffer 3 (20 mM HEPES, pH 7.8; 20 mM KCl; 1 mM $MgCl_2$; 2 mM DTT; 17% glycerol; and 0.2 mM PMSF) and assayed for protein content using a colorimetric assay. Aliquots were stored at −80°C until use.

Electromobility gel shift assay

Fifteen micrograms nuclear extract were incubated for 10 min with 1 μg poly(dI-dC), in 7.5% (v/v) glycerol, 38 mM KCl, and 0.6 mM $MgCl_2$. Oligonucleotides corresponding to the binding sites for NF-AT, NF-κB, and AP-1 were end labeled using T4 polynucleotide kinase. Incubation was performed at room temperature for 20 min before loading onto a 5% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was dried and exposed by autoradiography.

The oligonucleotides used were as follows: NF-AT, 5′ GGA GGA AAA ACT GTT TCA TAC AGA AGG CGT 3′; NF-κB, 5′ AGT TGA GGG GAC TTT CCC AGG C 3′; and AP-1, 5′ CGC AAG TGA CTC AGC GCG 3′.

Acidic sphingomyelinase assay

T cell blasts (6 × 10^6) were incubated for 1 h with either 1 mM chloroquine or a PBS vehicle control. Cells were then stimulated at 37°C for the indicated time points with fixed B7 transfectants at a 1:3 ratio of transfectants to T cells. Reactions were stopped by immersion in liquid nitrogen and subsequently pelleted in a microfuge. Pellets were lysed in 200 μl of 0.5% Nonidet P-40 (containing protease inhibitors as described above), and the supernatants were recovered. Protein (30–50 μg) was then incubated in a 50-μl reaction containing 250 mM sodium acetate and 1 mM EDTA, pH 5.0, containing 1.1 μCi/ml [^{14}C]sphingomyelin. After incubation for 2 h at 37°C, the reaction was phase separated by the addition of 200 μl H_2O and 800 μl of chloroform/methanol (2/1). The aqueous phase was then measured by scintillation counting. The procedure at each time point was conducted in triplicate, and the data were normalized relative to the zero time control as 100%. A minimum of three experiments were conducted in each case.

Results

CD28 stimulates proliferation in activated, but not resting, T cells

The requirement for CD28 costimulation in T cell activation has been established in numerous experiments, using both stimulatory and inhibitory reagents (1, 19, 20). In general, most investigations

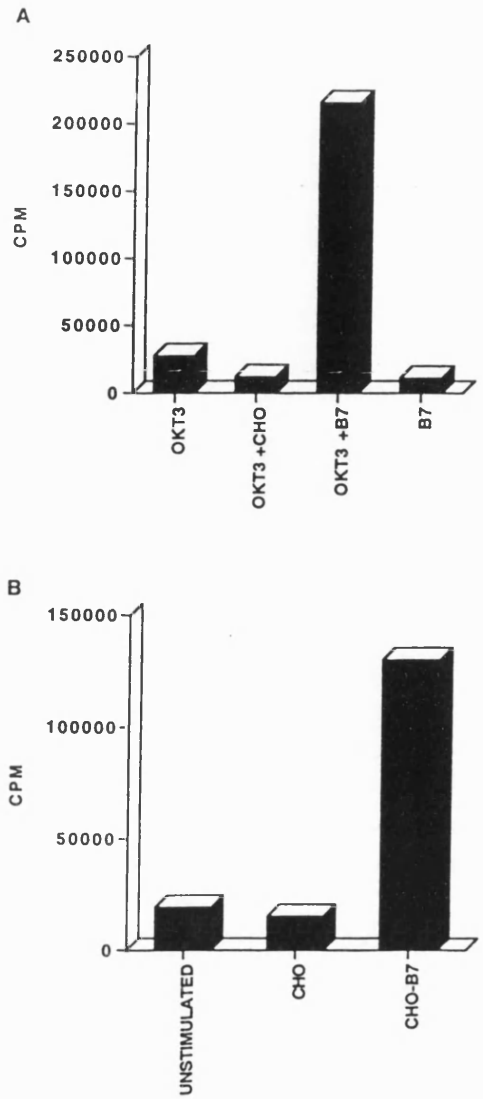

FIGURE 1. T cell blasts proliferate in response to B7 stimulation only. *A*, Purified peripheral blood T cells were stimulated with soluble anti-CD3 (mAb OKT3, 1 μg/ml), alone or in the presence of glutaraldehyde-fixed CHO or CHO-B7-transfected cells or with CHO-B7 cells alone in 96-well plates for 72 h. *B*, Day 8 SEA-T cell blasts were incubated in medium or with fixed CHO or CHO-B7 cells for 72 h. [^{3}H]Thymidine (1 μCi/well) was added, and the cells were harvested after an additional 18 h. Proliferation was measured by scintillation counting.

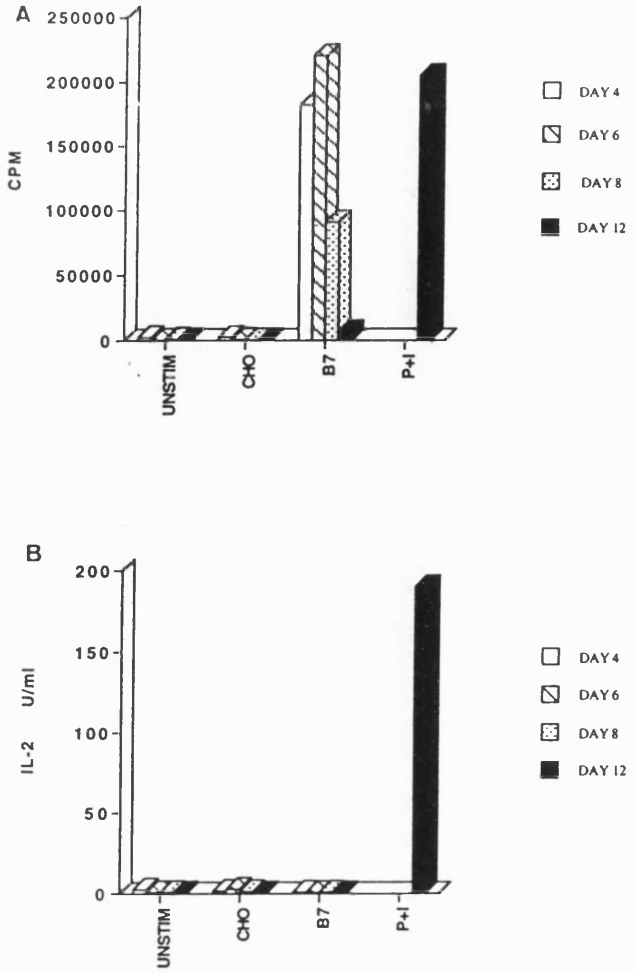

FIGURE 2. Kinetics of B7 responses in T cell blasts. SEA-stimulated T cell blasts were taken from culture at the the times shown post-antigenic stimulation, washed, and incubated in fresh medium alone (UNSTIM) in the presence of fixed CHO or CHO-B7 transfectants or with PMA (0.3 ng/ml) and ionomycin (1 μM) for 24 h (P+I). Proliferation was measured by [^{3}H]thymidine incorporation (*A*). Aliquots of supernatant were removed from the proliferation cultures and analyzed for IL-2 by CTLL bioassay (*B*).

have provided information on the requirements for CD28 costimulation in initiating the proliferation in resting T cells, but little is known concerning the role played by CD28 in maintaining this response. We were interested in establishing whether CD28 stimulation played a part in sustaining proliferation in normal T cells. Using transfectants expressing the CD28 ligand B7-1 (CD80) as a source of costimulation, we compared the effects of CD28 engagement in both resting and activated T cells to determine whether CD28 ligation could further stimulate previously activated cells. Consistent with previous findings, we observed that CD28 costimulation was absolutely required to achieve optimal proliferative responses to anti-CD3 Abs in resting T cells (Fig. 1*A*) and that neither anti-CD3 nor B7 stimulation alone was sufficient to stimulate a response. In contrast, in experiments using T cells previously activated by superantigen stimulation (T cell blasts), a clear proliferative response was observed following CD28 stimulation alone (Fig. 1*B*). This experiment demonstrated that CD28 was capable of transmitting proliferative signals to activated T cells, but not to T cells in a resting state. This finding was similar to that of Linsley et al., who also observed the ability of B7 to directly stimulate PHA blasts (1). To determine whether these responses by T cell blasts were down-regulated as the T cells became more quiescent, responses were investigated at various time points following antigenic stimulation. This experiment (Fig. 2) demonstrated that the response to B7 stimulation by activated T cells was dependent upon the state of activation. Up to 8 days following antigenic stimulation, CD28 engagement induced a substantial proliferative response; however, as the cells became more quiescent, the CD28 response diminished, causing minimal proliferation by day 12 (Fig. 2*A*). In the course of these experiments we also observed that by day 12 T cells were no longer responsive to either CD3 or CD28 alone and had reestablished the requirement for both signals to induce proliferation. This, therefore, established that substantial differences existed in the responses of resting and activated T cells to CD28 engagement, but that these differences

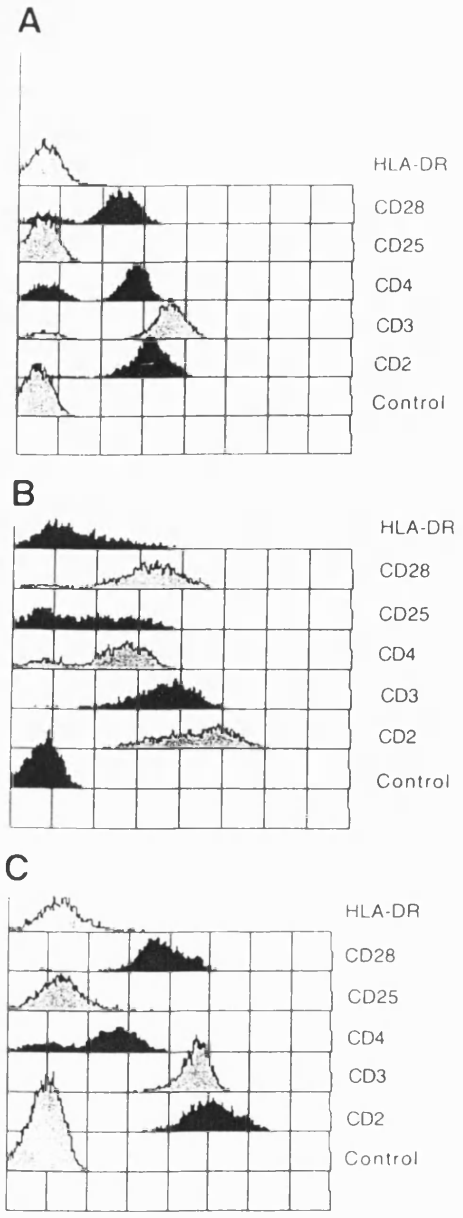

FIGURE 3. FACS analysis of the T cell populations used. T cells were analyzed on day 0 (*A*), day 6 after SEA stimulation (*B*), and day 12 after stimulation (*C*) and stained for the markers shown.

FIGURE 4. Detection of transcription factor complexes in day 6 T cell blasts. SEA-activated T cells were analyzed for the presence of NF-AT and AP-1 6 days following SEA stimulation. The cells were unstimulated (U), stimulated with PMA and ionomycin (P/I), or stimulated with CHO-B7 expressing transfectants (B7). Nuclear extracts were prepared, and the complexes were separated on a 5% acrylamide gel and visualized by autoradiography.

were transient, and ultimately, T cells returned to having the activation requirements of resting T cells. In addition to investigating the proliferative response of B7-stimulated T cell blasts, the effect on IL-2 production was studied. Surprisingly, we were unable to detect any significant levels of IL-2 production in the B7-stimulated T cell blasts measured by CTLL bioassay (Fig. 2*B*) despite the induction of substantial proliferation. To ensure that this lack of IL-2 production was not associated with either an incapacity to make IL-2 or a lack of cellular viability, T cells were fully restimulated with PMA and ionomycin and assessed for both proliferation and IL-2 production on day 12 (Fig. 2, *A* and *B*). Since these responses were fully intact, our data suggested that normal activated T cells could respond to CD28 stimulation in isolation for a period of time following antigenic stimulation, and that while this response was not characterized by significant IL-2 production, the cells could generate proliferative responses. Notably, both IL-2 and proliferative responses could readily be stimulated by subsequent full activation, suggesting that a limited set of signals was provided by CD28 under these conditions, which could stimulate proliferation but not the large amounts of IL-2 seen when fully activating the cells.

The surface phenotype of the T cells used in these experiments was also established by FACS analysis at various time points. These data (Fig. 3) showed that the resting T cells were predominately $CD3^+$ $CD25^-$ and $HLA\text{-}DR^-$ (Fig. 3*A*), consistent with their resting status. In contrast, activation with superantigens resulted in a significant up-regulation measured on day 6 of CD2, CD25, and HLA-DR molecules, as expected (Fig. 3*B*); and by day 12, as the cells became more quiescent, it was evident that activation markers, in particular CD25, were down-regulated (Fig. 3*C*). In addition to these markers, we examined T cells for the presence of CTLA-4 and CD80 expression, but we did not detect either of these markers on our activated T cells.

CD28 stimulation directly results in the generation of NF-κB and AP-1, but not NF-AT

Following the observation that B7 engagement was sufficient to stimulate proliferation in T cell blasts, we sought to establish which signaling events, such as the generation of transcription factor complexes, might be involved. We, therefore, measured the induction of transcription factors known to be important in T cell proliferation (NF-AT, AP-1, and NF-κB) using electrophoretic mobility shift assays (gel shift assays). These experiments revealed a number of interesting features. Initially, we noted that our T cell blasts were highly active (up to day 6, as evidenced by proliferation assays), and that there were high levels of transcription factors, including NF-AT and AP-1, found in T cell blasts at this time, making measurement of responses to B7 stimulation difficult (Fig. 4). At these early time points, we were unable to detect increases in transcription factors in response to either PMA and ionomycin

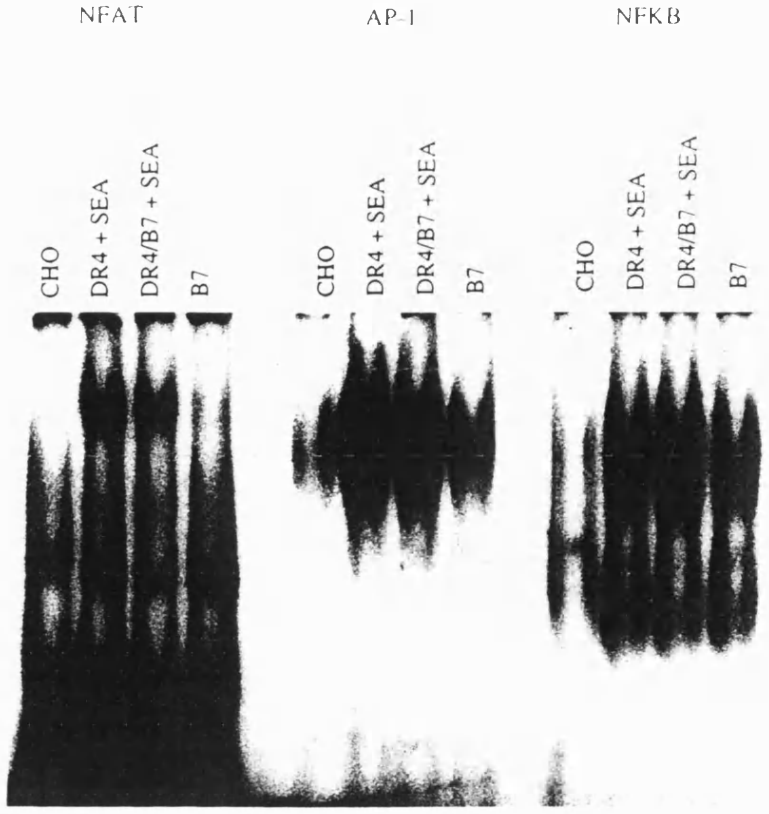

FIGURE 5. Induction of transcription factors in superantigen and B7-stimulated T cell blasts. Day 10 SEA T cell blasts were stimulated with fixed CHO cells, CHO-DR4 cells, or CHO-DR4/B7 cells pulsed with SEA or CHO-B7 cells alone for 4 h. Nuclear extracts were prepared and incubated with ^{32}P-labeled oligonucleotides representing consensus NF-AT, AP-1, and NF-κB binding sites. Complexes were separated on a 5% acrylamide gel and visualized by autoradiography.

or with B7. Therefore, to study the induction of transcription factors in response to B7 stimulation, we allowed the T cells to quiesce further, and experiments were routinely performed on day 10 post-SEA stimulation. By using T cell blasts at later time points, we observed substantially lower levels of transcription factors in unstimulated cells.

At these later time points (Fig. 5), we observed that stimulation of T cells with either DR-4 or DR4-B7 transfectants pulsed with SEA resulted in substantial up-regulation of all three factors measured (NF-AT, AP-1, and NF-κB). Firstly, this clearly demonstrated a requirement for TCR stimulation in the induction of NF-AT in this system, and furthermore, measurements of IL-2 production from the same experiment (Fig. 6) showed a strong correlation between the ability of T cells to make IL-2 and the induction of NF-AT. Thus, superantigen stimulation of the TCR alone was sufficient to induce both proliferation and IL-2 production in T cell blasts. However, CD28 costimulation in addition to superantigen stimulation considerably augmented IL-2 production, demonstrating that additional costimulation had indeed occurred.

In addition to the above events, we observed that T cells challenged with B7-expressing transfectants alone responded by the up-regulation of both AP-1 and NF-κB; however, stimulation by B7 alone did not stimulate the generation of either NF-AT or IL-2. These data demonstrated that NF-κB and AP-1 were downstream targets of CD28 stimulation and that while these transcription factors were not exclusive to CD28 signaling, NF-κB and AP-1 were significant targets of CD28 signaling. Furthermore, our data confirmed previous observations on the involvement of TCR pathways in the induction of NF-AT and IL-2.

CD28 signaling involves both PI3K and acidic sphingomyelinase activation

Two pathways that have been suggested to be involved in CD28 signaling are those via PI3K and acidic sphingomyelinase. We, therefore, sought to establish whether either of these pathways was being used following B7 stimulation of the T cell blasts. We have previously demonstrated that in response to B7 stimulation, PI3K is both recruited and activated by CD28 (5); using a similar approach in this system, we observed that B7 stimulation resulted in PI3K recruitment by CD28 (data not shown). To establish whether CD28 stimulation could also activate the acidic sphingomyelinase pathway in T cell blasts, as previously reported, the effect of B7 stimulation on this pathway was also measured (Fig. 7). This analysis revealed that following B7 engagement there was a rapid elevation in sphingomyelinase activity in B7-stimulated, but not control, lysates, indicating that CD28 ligation in these T cell blasts could lead to the activation of sphingomyelinase activity. Furthermore, this activity could be inhibited by the use of chloroquine, which is reported to inhibit the activation of acidic sphingomyelinase. Thus, we had evidence that both PI3K and acidic sphingomyelinase pathways were being activated following the engagement of CD28 in isolation in our T cell blasts.

Having demonstrated that both acidic sphingomyelinase and PI3K pathways were activated by CD28 in the absence of TCR engagement, gel shift experiments were performed using chloroquine and wortmannin to investigate whether these pathways were involved in NF-κB and AP-1 generation. These results revealed that pretreatment of the T cell blasts with wortmannin produced substantial, but not complete, inhibition of AP-1 generation (Fig. 8*A*). In addition, in some experiments chloroquine had an effect on AP-1 induction, but this effect varied between cultures (compare Fig. 8, *A* and *B*), raising the possibility that AP-1 may be a downstream target of both PI3K and acidic sphingomyelinase pathways. In contrast, NF-κB induction was not inhibited by wortmannin; in fact, it showed a slight increase compared with that in the B7 control. This observation is interesting in light of the fact that wortmannin has been suggested to result in increased IL-2 production from some T cells (21), and this may be one possible

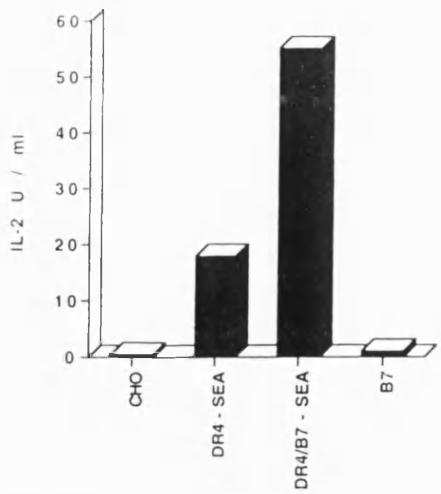

FIGURE 6. Corresponding IL-2 production in T cell blasts stimulated as shown for the gel shift assay (Fig. 5). Stimulated cell aliquots (5×10^4) were removed before preparation of the nuclear extracts and incubated in 96-well plates for an additional 24 h. Supernatants were removed, and IL-2 production was assessed by CTLL assay.

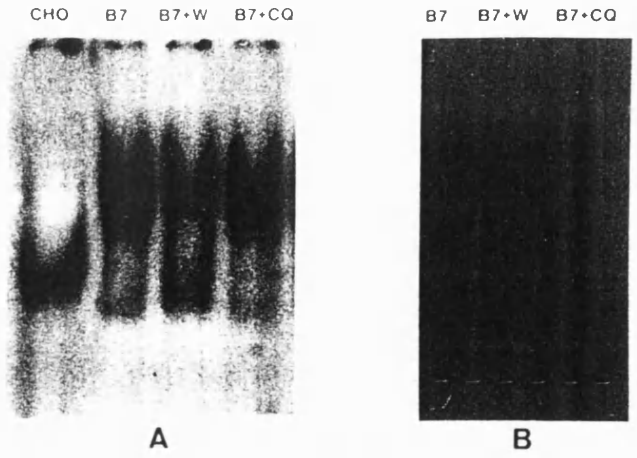

FIGURE 8. Effects of wortmannin and chloroquine on B7-induced transcription factor generation. Day 10 T cell blasts were incubated in medium alone or in the presence of wortmannin (100 nM) or chloroquine (50 μM) for 1 h before a 4-h stimulation with fixed CHO or CHO-B7 transfectants. Nuclear extracts were prepared and analyzed for AP-1-binding activity in two different experiments (*A* and *B*).

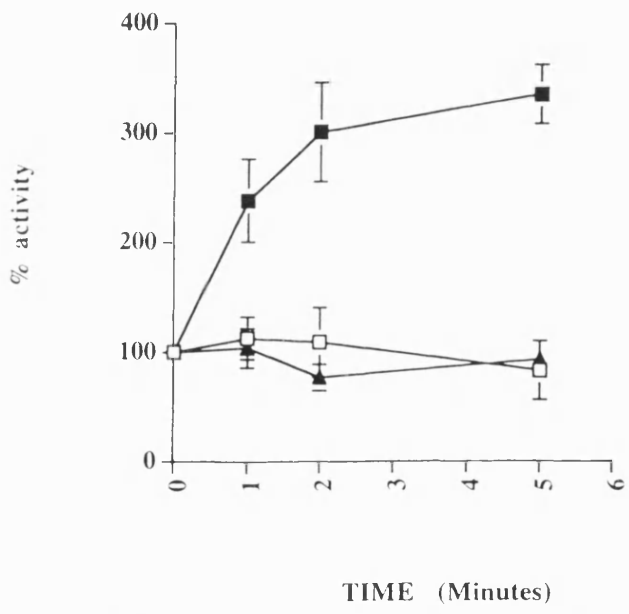

FIGURE 7. Effect of chloroquine on acidic sphingomyelinase activity in B7-stimulated T cell blasts. Day 10 T cell blasts were preincubated for 1 h in medium (closed symbols) or with 1 mM chloroquine (open symbols). After washing, the cells were stimulated with fixed CHO (triangles) or CHO-B7 (squares) cells for the time specified. Cytoplasmic cell extracts were prepared and analyzed for enzymatic activity on [^{14}C]sphingomyelin. Results are shown as the percentage of activity at time zero.

FIGURE 9. Effects of wortmannin and chloroquine on NF-κB. Day 10 SEA blasts were treated with 100 nM wortmannin (B7 + W) or 50 μM chloroquine (B7 + CQ) for 1 h before a 4-h stimulation with fixed CHO or CHO-B7 transfectants. Nuclear extracts were prepared and analyzed as described in *Materials and Methods*.

mechanism. In contrast, chloroquine completely inhibited the induction of NF-κB in response to CD28 stimulation (Fig. 9), providing a strong indication that CD28 induction of NF-κB may be mediated via acidic sphingomyelinase.

Overall, therefore, these results suggested that both the PI3K and acidic sphingomyelinase pathways were activated by CD28 stimulation, and inhibition of either of these pathways could affect the generation of NF-κB and AP-1 transcription factor complexes.

Discussion

CD28 costimulation is a critical event in T cell activation. In its absence, resting T cells do not fully enter the cell cycle or proliferate in response to TCR engagement, and T cells may enter either anergic or apoptotic states (22–24). We have studied a system in which activated T cells were found to be responsive to CD28 stimulation alone, and we have used this system to better characterize

CD28 signals as distinct from those provided by TCR stimuli or mitogenic agents. The proliferative effects of B7 (CD80) stimulation were detectable in T cell blasts up to 8 days following antigenic challenge. This response was not due to the persistence of TCR stimulation by Ag in our cultures, since we were unable to observe either NF-AT or IL-2 production in response to B7 alone, whereas both were demonstrable following TCR engagement with superantigen. This indicated that the signals arising from the CD28 ligation resulted directly in the generation of the transcription factors AP-1 and NF-κB.

The observation that generation of NF-AT required TCR engagement and correlated with the production of IL-2 suggests that this model of T cell stimulation is compatible with that in previous studies (25, 26) and confirms the critical role of TCR stimulation in IL-2 production. Somewhat surprisingly, NF-AT, IL-2 production, and proliferation were observed in experiments using DR4-SEA complexes alone, in contrast to our experience of proliferative responses to anti-CD3 that require CD28 costimulation. Taken in conjunction with the observation that MHC-superantigen complexes can also stimulate proliferation in resting T cells (19), this indicates that superantigens may be more capable of providing costimulatory signals, in line with previous suggestions (27). Furthermore, the up-regulation of both NF-κB and AP-1 by DR4-SEA stimulation implies activation of costimulatory pathways, since activation of these factors has been linked to ceramide (14) and JNK pathways (28), neither of which results directly from TCR stimulation alone, and both of which can now be linked to costimulatory pathways such as CD28.

The present data also confirm that considerable differences exist in the interpretation of CD28 signals by resting and activated T cells. In particular, while CD28 ligation alone is capable of stimulating proliferation in activated T cells, this response is not observed in resting cells despite the fact that both PI3K and acidic sphingomyelinase interactions have been observed (5, 6). This presumably indicates that in resting T cells the pathways activated by the TCR are critical in allowing proliferation to proceed, and these pathways remain open in activated T cells for some time, thus allowing CD28 signals to induce proliferation. Perhaps more surprisingly, we also noted that B7-induced proliferation in T cell blasts was not associated with IL-2 production. This is in contrast to the significant amounts of IL-2 observed following CD28 costimulation of the TCR and indicates that IL-2 production is stringently regulated and correlates well with NF-AT production. Furthermore, our data indicate that T cells can respond by proliferation in the absence of detectable IL-2 when given suboptimal stimuli such as CD28 alone. However, we did observe a B7-dependent increase in IL-2 output when T cells were stimulated with DR4/B7-SEA compared with DR4-SEA, demonstrating that costimulation was provided by B7 in these circumstances. Since transcription factor levels induced by DR4/B7-SEA compared with those induced by DR4-SEA were indistinguishable, the additional IL-2 production observed in the presence of B7 may, therefore, reflect increased transcriptional activity or mRNA stabilization by CD28, as has been reported previously (17).

One transcription factor complex reported to be induced by CD28 is the CD28 response complex (CD28RC) (18), which binds to a conserved DNA sequence within the IL-2 promoter. More recent investigations of this complex, however, have shown that it can be induced in the absence of CD28 receptor engagement by mitogenic agents (29), and that the complex comprises members of the Rel family of NF-κB-related proteins (30, 31). Consistent with this is the fact that in the presence of PMA, CD28 has been reported to have an effect on NF-κB induction via the down-regulation of its cytoplasmic inhibitor, IκB (29, 32). Our data are consistent with these reports, in that we found NF-κB to be a target of CD28 costimulation. Furthermore, this response was sensitive to inhibition by chloroquine, but not by wortmannin, indicating that NF-κB may be primarily downstream of acidic sphingomyelinase in CD28 signaling. In line with this finding, previous reports have shown that other receptors, such as TNF, that activate sphingomyelinase can also induce NF-κB (6, 7).

We have also identified AP-1 as a downstream target of CD28 signaling. AP-1 generation has previously been shown to involve the activation of c-*jun* kinase (JNK) following TCR and CD28 receptor stimulation (28). However, while Su et al. demonstrated a requirement for both TCR and CD28 signals in JNK activation, our observation that CD28 could induce AP-1 activity in the absence of TCR engagement may be explained by the fact that our work was performed in activated T cell blasts compared with Jurkat cells. Currently, the receptor proximal events that are triggered by CD28 in the activation of JNK are unclear. However, since the acidic sphingomyelinase and PI3K pathways have both been shown to be associated with CD28 signaling, the involvement of one or possibly both of these pathways is implicated in the CD28-mediated activation of JNK.

Previously, we identified activation of the PI3K pathway in response to CD28 engagement (5). However, while recruitment and activation of this enzyme by CD28 are unequivocal, there are discrepancies concerning the importance of this pathway in stimulating IL-2 production (5, 33, 34). In resting T cells, blocking of PI3K activity by wortmannin results in inhibition of both proliferation and IL-2 production, whereas in activated cells such as Jurkat cells, no inhibition is seen, and there may be an increase in IL-2 output (21), implying a negative regulatory role for PI3K. Furthermore, while PI3K has been suggested to be involved in the induction of JNK and AP-1 via the small GTPases, Rac1 and Cdc42 (35, 36), the inability of wortmannin to completely inhibit AP-1 induction in our studies indicates that additional pathways may be involved. Interestingly, we also observed during these studies that chloroquine had variable inhibitory effects on AP-1 expression, raising the possibility that activation of acidic sphingomyelinase provides an alternative route to AP-1 following CD28 stimulation in some circumstances. This possibility would explain the inability of wortmannin to completely inhibit AP-1 generation as well as the effects of chloroquine on AP-1. Furthermore, such a hypothesis is consistent with data indicating that other receptors linked to acidic sphingomyelinase/ceramide pathways, including TNF, can activate JNK (13, 37).

Since it appears that both AP-1 and NF-κB might be activated via the acidic sphingomyelinase pathway, what then is the role of PI3K? Recent reports have implicated PI3K in the activation of the signaling molecule PKB (38) and the cell cycle regulator S6 kinase (39). Interestingly, S6 kinase has also been shown to be activated by CD28 and is wortmannin sensitive. Thus, one major role for PI3K may be in the promotion of cell cycle progression or, possibly, T cell survival. Evidence that PI3K may prevent apoptosis has been suggested in other cell systems (40) and may play a role in T cells. This would be consistent with the functional role of CD28 in preventing anergy and apoptosis in response to TCR engagement (23, 41).

The requirement for CD28 in the activation of T cells has been repeatedly demonstrated; however, the signals that mediate these effects are less well defined. The present studies indicate that both AP-1 and NF-κB are direct targets for CD28 signals and that both PI3K and acidic sphingomyelinase activation are involved in their generation. While it is likely that there are other effects mediated by the recruitment of tyrosine kinases to CD28, such as *lck*, *fyn*,

and *itk* (42–44), our data establish an important role for both acidic sphingomyelinase and PI3K in CD28 signaling.

Acknowledgments

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